The interaction of platelets and exogenous antigenic factors in the causation of atherosclerosis and coronary heart disease

Thesis

THE INTERACTION OF PLATELETS AND EXOGENOUS ANTIGENIC FACTORS IN THE CAUSATION OF ATHEROSCLEROSIS AND CORONARY HEART DISEASE

by

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CONTENTS

ABSTRACT

The Interaction of Platelets and Exogenous Antigenic Factors in the Causation of Atherosclerosis and Coronary Heart Disease

This work describes aspects of the role of platelets in the development of atherosclerosis and coronary heart disease (CHD). Emphasis is on the involvement of "young" platelets, which are metabolically more active than the other population of these cells and are consequently termed "active" platelets. Aggregation investigations were used to detect an increase in this fraction of the platelet population and electron microscope studies confirmed that these aggregation results reflected the total activity of the platelets. Surveys performed in conjunction with the Medical Research Council Epidemiology Unit showed abnormal results in approximately 18% of the normal population and in approximately 90% of CHD patients. This latter group was tested at least twelve months after recovery from the heart attack (myocardial infarction).

There are immunological factors which may be important and are investigated in this work. The involvement of these factors are indicated by the report relating antibody to whole dried milk with CHD. This original observation was confirmed in surveys performed in collaboration with the Medical Research Council Epidemiology Unit. Because of the large number of specimens involved in these surveys, an automated method was developed. This method was also used to survey the distribution of food antibodies in the normal population.

It was found that the milk antibody originally related to CHD was produced in response to an antigen present in the cream portion of milk. It was determined that the antigen resided in the bovine milk-fat globule-membrane. Because it has previously been shown that cross-reactivity exists between membranes of different species, the possibility

of antibody to bovine milk-fat globule-membrane reacting with human platelets was considered and the resulting investigations, which demonstrated this occurrence, are described.

The possibility is considered that increased platelet turnover in this disease, with an increased percentage of "active" platelets, may well be the result of the action of antibody to bovine milk-fat globule-membrane. Interaction between membranes may therefore play a role in the development of atherosclerosis.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

This thesis discusses and examines the role of platelets in the development of coronary heart disease (CHD). The involvement of certain immunological factors is also described.

There is evidence to show that a higher percentage than normal of "active" platelets are present in the circulation of hospital-based CHD patients in the acute phase of the disease. Controversy remains regarding their role and significance and their presence in the circulation has been attributed to the action of atheromatous tissue and subsequent ulceration. The work described in this thesis investigates the platelet activity in "long-term" CHD patients and also in apparently healthy control subjects. It also examines the hypothesis that an increased percentage of "active" platelets in the circulation could be one important contributory factor responsible for initiating the process which eventually leads to atherosclerosis.

The immunological aspect of CHD concentrates on the reported involvement of antibodies to whole dried milk in this condition. An experimental programme was designed to elucidate the role of this phenomenon in CHD. The results obtained are reported and discussed.

The involvement of the platelets and the new immunological approach to CHD are detailed in the following sections.

1.1.1 The platelet-morphology and function. In the circulating blood the platelets appear as disc-shaped cells with an average longitudinal diameter of approximately 2-3μm (1) and 0.5-1μm in thickness (2). An historical review by Tovantins (3) describes how the platelets were recognised as cells early in the first quarter of the 19th century but most workers failed

to appreciate their importance until Bizzozero in 1882 (4) related their function to the coagulation of blood. Wright in 1910 (5) established that platelets are detached portions of the cytoplasm of a cell produced in the bone marrow called the megakaryocyte. Although megakaryocytes are frequently found in the lungs (6), in the marrow they mature from the multipotential stem cell which differentiates into the erythrocytic, myelocytic and megakaryocitic series (7). The earliest recognisable member of the megakaryocytic series is the megakaryoblast. In contrast to other cells these divide by a process termed endomitosis (8), a term describing nuclear division with the absence of cytoplasmic division. This cell develops into the promegakaryocyte and subsequently into the mature megakaryocyte. In the marrow, these cells are situated close to the sinusoidal membrane (9) and Wintrobe (10) describes how, from the megakaryocyte "platelet formation begins by the development of numerous long cytoplasmic pseudopods that extend through apertures in the sinus membrane into the lumen, much in the manner of an octopus. These pseudopodia become thinner and ultimately filiform and granular masses, the size of platelets, form within them. These are probably caught in sinusoidal capillaries, segments are broken off by their contraction, and the platelets thus formed are swept away by the blood stream". It has been calculated (11, 12) that one megakaryocyte is capable of releasing between 2,000 and 7,000 platelets.

The mechanism that controls the turnover of platelets in humans has not been fully explained (13), however it has been postulated that the hormone called thrombopoietin regulates the release of the platelets from the megakaryocytes (14, 15). It is not clear what stimulus is responsible for the action of this hormone, but it has been suggested that it may depend more on the total body content of platelets than upon their concentration in the blood (16, 17). It has been estimated that the platelet turnover rate in normal subjects is 35,000 ± 4,300 platelets/µl/day (18) and the platelet count in adults averages 250 x 10^9/l and ranges from 140-440 x 10^9/l (10).

The normal life-span of the platelet, in normal subjects,

has been reported to be between 9 and 12 days (19-21). Shulman and his co-workers (22) demonstrated that after leaving the marrow the platelets are stored in the spleen for approximately two days. On leaving the spleen the cells remain in the circulation for approximately 10 days. During this time it appears that a proportion of the circulating platelets enter a splenic pool where they are exchanged with new cells, which in turn, re-enter the circulation. Although Shulman (22) states that approximately one third of the platelet are sequestrated in the spleen, Aster and Jandle (20) demonstrated that the majority of circulating platelets are destroyed by the liver. Controversy remains regarding the exact mechanism controlling the destruction of the platelets and it appears that platelets are removed from the blood stream by random destruction (23) and senescence (24). The newly-formed platelets released from the spleen appear to be metabolically more active than the other platelets present in the circulation (22, 25-30) but it has been suggested that these cells only show this activity for approximately two days (22). Section 1.1.3 (page 15) of this thesis examines the relationship between an increase in the percentage of these platelets and the development of coronary heart disease.

The major function of the platelet is in haemostasis (31) which is a process in which the cell plays both a mechanical and biochemical role. Wintrobe (10) describes how vascular injury results in platelets adhering to the vessel wall, an action initiated by the exposed collagen fibres. Such adherent platelets release stored ADP (the release reaction) which results in the formation of further platelet aggregates. The activation of certain platelet factors and the initiation of blood coagulation factors then follow, leading to the consolidation of the platelet plug by fibrin and subsequently to the phenomenon of clot retraction. In addition to their role in the process of coagulation, platelets act to maintain or support the vascular integrity of the vasculature by attaching to gaps which normally develop in the endothelium and as a result they actually enter and are incorporated into endothelial cells (32-35). This phenomenon is illustrated in Fig. 1.

Fig. 1. Diagram of the mechanism involved when a platelet enters the endothelial cytoplasm.

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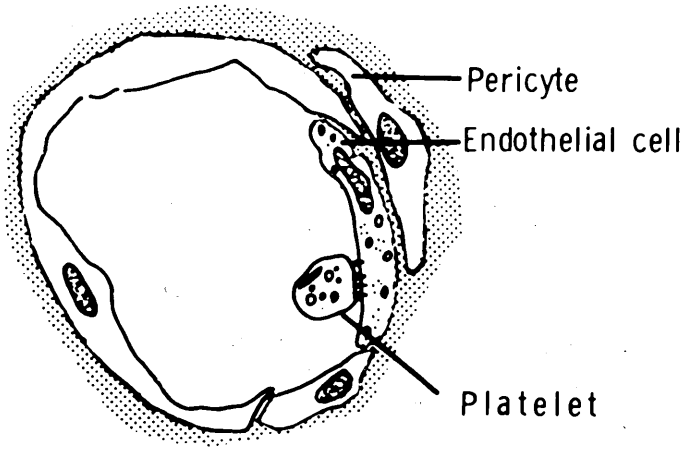
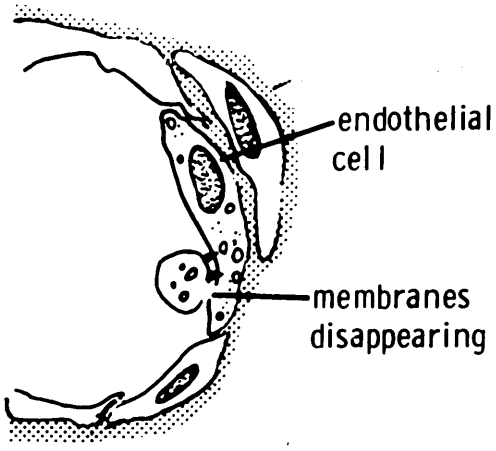
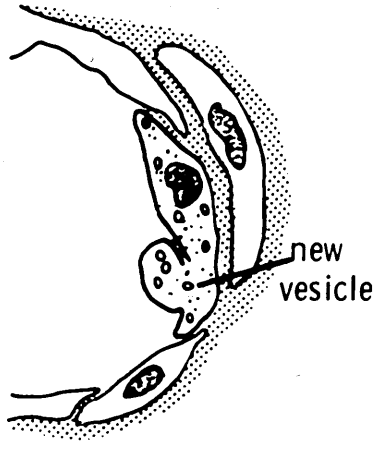
From Johnson, S. A. (1971), p.296, (13).



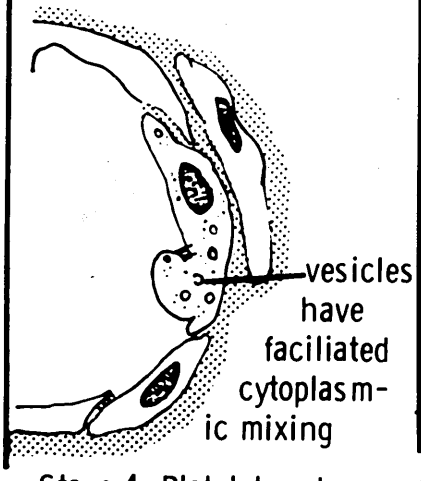
Stage 1: Platelet and endothelial membrane form dense areas



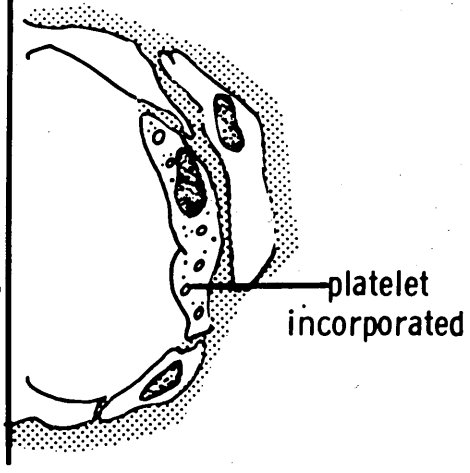
Stage 2: Portions of platelet and endothelial membranes disappear



Stage 3: Vesicles form at membrane areas



Stage 4: Platelet and endothelial contents mix



Stage 5: Platelet identity lost

Electron microscopy studies have shown that platelets are far more complex than suggested by their deceptively simple appearance when viewed by light or phase contrast microscopy. The ultastructure of the platelet is illustrated in Fig. 2.

Platelet anatomy can be divided into three main functional divisions:-

(a) the peripheral zone which includes the exterior coat, unit membrane and submembrane area. This zone is responsible for adhesion and aggregation.

(b) The sol-gel zone which contains the microtubules and the microfilaments. Their main function is contraction and support.

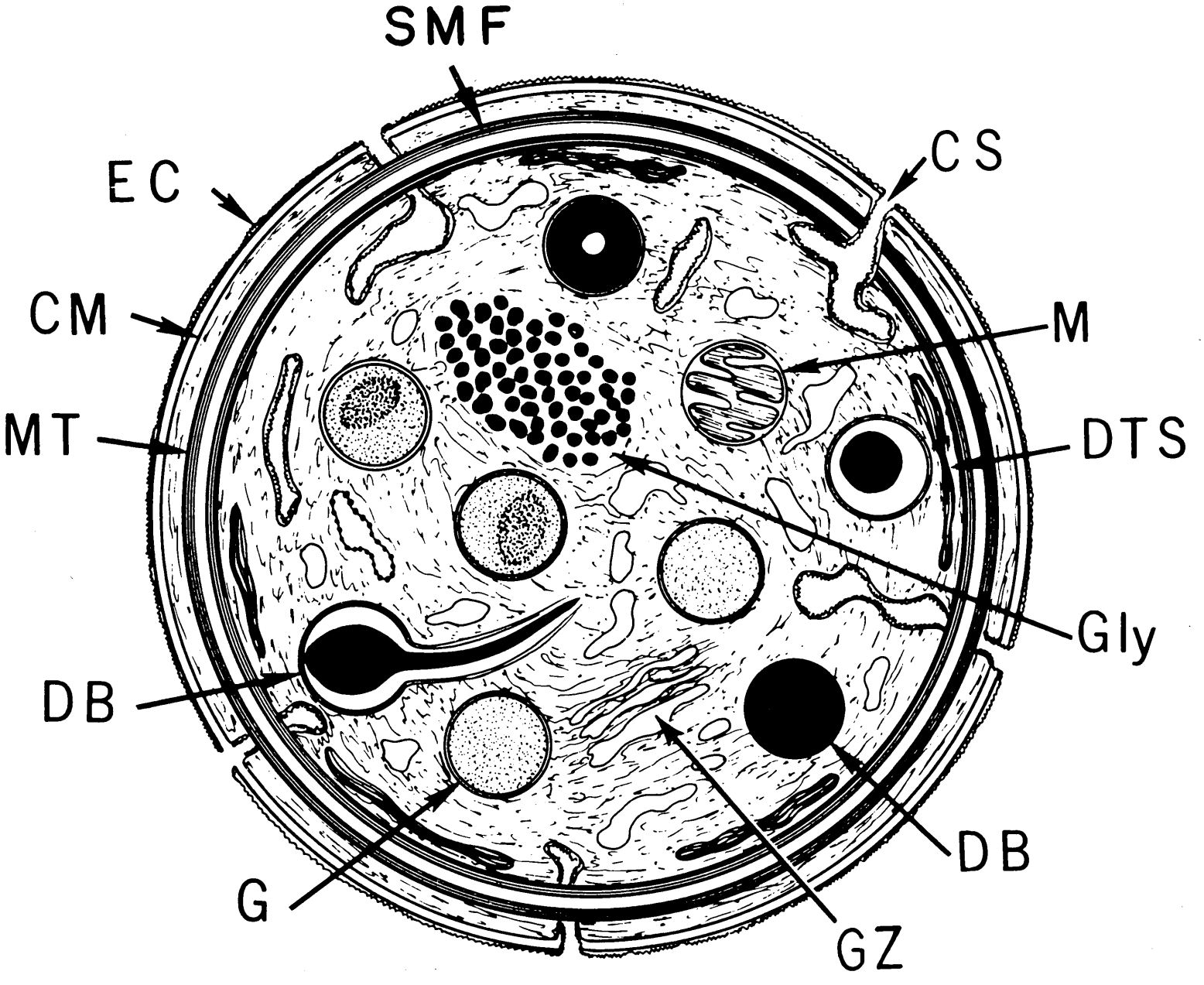
(c) The organelle zone containing the granules, dense bodies and mitochondria whose main function is storage and secretion.

(a) The peripheral zone is the interface between the platelet and the plasma (13). The outer "coat" surrounding the surface of the platelet is approximately 10-20nm thick and is referred to as the "fluffy coat" or plasmatic atmosphere. The exact composition of this "fluffy coat" is not known, but it is considered to be important in the adhesion and aggregation of platelets (1).

"Adhesion" is the term used to describe the process by which platelets attach themselves to a non-platelet surface (36) and "aggregation" describes the attachment of platelets to each other (10). In vivo, platelets will adhere to collagen fragments, basement membrane and microfibrils which are associated with elastin (2, 37). Mayer (38) describes how both physical and chemical determinants are present on collagen fibres which together are responsible for platelet adhesion leading to platelet activation. The main physical factor involved is the triple helical shape of the collagen fibres and it appears that the chemical determinants recognised by the platelets on these triple helical surfaces involve some general features of

Fig. 2. Diagramatic representation of platelet anatomy. Included is the peripheral zone, the sol-gel zone and the organelle zone. Structural elements of the peripheral zone include the exterior coat (fluffy coat) (EC), the unit membrane (CM) and a submembrane area containing submembrane filaments (SMF). The latter are also included with the circumferential band of microtubules (MT) and microfilaments as the three major fibre systems constituting the matrix of the sol-gel-zone. Granules (G), dense bodies (DB), and mitochondria (M) are the formed organelle zone. The open canalicular system (CS) and the dense tubular system (DTS) are also illustrated together with a Golgi zone (GZ) present in approximately 10% of platelets. Glycogen particles (Gly) are usually concentrated in masses on the metrix.

From Johnson, S. A. (1971), p.48, (13).


SMF
EC
CS
CM
M
MT
DTS
Gly
DB
G
GZ
DB

collagen composition, in particular the high proline/hydroxyproline content. This complex adhesion mechanism has also been ascribed to neuraminidase in the platelet membrane which exposes acceptor groups in the collagen by hydrolising sialic acid residues (39). The groups exposed in the collagen react with glycosyl transferases located in the platelet membrane (40).

Following adhesion, the platelets undergo a secretory process by which substances found in the dense bodies and the alpha granules are secreted. Grette (41) named this process, "the release reaction" and the ADP released results in the formation of the platelet aggregate. Aggregates became apparent in experimental wounds within 15 seconds of the adhesion process (2). In vitro, platelet aggregation may be observed by an instrument called a "platelet aggregometer" (42). This instrument is a photo-optical instrument which is connected to a strip recorder chart. Platelet-rich plasma, which is turbid, is stirred in a cuvette and the transmittance of light through the sample, relative to a platelet-poor blank, is recorded. When an aggregating agent is added, the formation of increasingly large platelet aggregates is accompanied by a clearing in the platelet rich plasma, and therefore, light transmittance through the sample is increased. The light received through the sample is converted into electronic signals, amplified and recorded on the chart recorder paper (Fig. 3).

The substances that induce platelet aggregation are many and varied. They are listed in Table 1. Platelet aggregation proceeds through three definite recognisable phases (1):-

(a) the change of shape of the cells after stimulus with the aggregating agent.

(b) primary reversible aggregation as the result of the stimuli.

(c) secondary or irreversible release action dependent on the concentration of the aggregating agent.

Fig. 3 showing typical aggregation curves produced by various aggregating agents.

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From Triplett, D. A. et. al. (1978), p.114 (1).

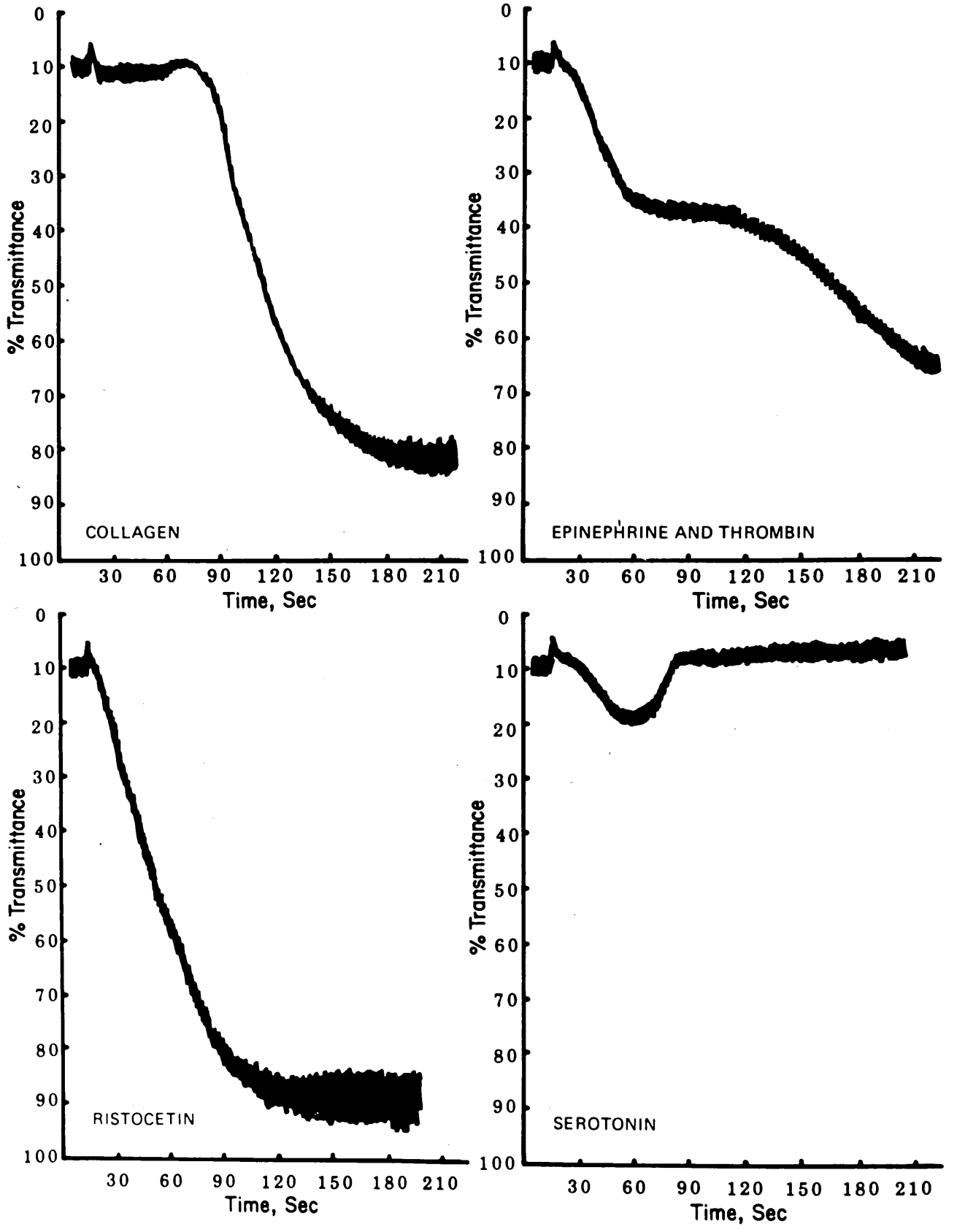
% Transmittance
0
10
20
30
40
50
60
70
80
90
100
COLLAGEN
30 60 90 120 150 180 210
Time, Sec
% Transmittance
EPINEPHRINE AND THROMBIN
Time, Sec
% Transmittance
RISTOCETIN
Time, Sec
% Transmittance
SEROTONIN
Time, Sec

Table 1 showing substances that induce release in the platelets.

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From Triplett, D. A. et. al. (1978), p.16 (1).

Inducer	Inducer Type	Shape Change	Adhesion/ Aggregation	Release I (Dense Body)	Release II (Alpha Granule)
Low molecular weight					
Adenosine diphosphate (ADP)	Weak	+	+	+	–
Epinephrine	Weak	–	+	+	–
Norepinephrine	Weak	+	–	+	–
Serotonin	Weak	+	–	–	–
Vasopressin	Weak	+	+	+	–
Proteolytic enzymes					
Thrombin	Strong	+	–	+	+
Trypsin	Strong	+	+	+	+
Snake venom	Strong	+	+	+	+
Papain	Strong	+	+	+	+
Particulate matter					
Collagen	Strong	+	+	+	+
Latex particles	Strong?	+	+	+	+
Fatty acids	Strong?	+	+	+	+?
Endotoxin	Strong?	+	+	+	+?
Thorium dioxide	Strong?	+	+	+	+?
Viruses	Strong?	+	+	+	+?
Antigen-antibody	Strong	+	+	+	+
Strongly positive-charged ions					
Polylysine	Strong	+	+	–	+
Lanthanum	Strong	+	+	–	+
Ristocetin	Strong	+	+	+	
Concanavalin A					
Bovine fibrinogen					
Distilled water					

The first recognisable sign of platelet activation is their change of shape into disc-spiny spheres. This change is induced by all aggregating agents except adrenaline (See Table 1). This process is thought to involve the uptake of water resulting in a 30% increase in platelet volume. This phenomenon takes a few seconds to occur at 37°C and it is possible that hidden adhesive sites on the platelet membrane are exposed during this phase of the platelet response (43).

When platelets are stimulated, they undergo reversible or irreversible aggregation depending on the concentration of the agent involved. The direct aggregation of platelets by ADP and other aggregating agents is called the primary, first phase or reversible aggregation. Aggregation that is irreversible is achieved through the release of the dense bodies and is referred to as secondary or second phase aggregation (1). This process involving the release of the contents of the dense bodies is termed "release I" and the extrusion of the alpha granules is termed "release II" (44).

The ADP store in the dense bodies accounts for approximately 60% of the total ADP in human platelets. This ADP is nonmetabolic or storage ADP, which is to be distinguished from the metabolic pool (45). This latter metabolic ADP actively participates in the metabolism of the blood platelets and provides energy for the maintenance of the membrane transport systems, various synthetic processes and the response to such stimuli as aggregation and retraction. Most platelet aggregating agents can produce the biphasic or irreversible response though this depends on the concentration of the inducing agent (See Table 1). It is considered that this irreversible response is the result of the aggregation induced by the ADP released from the dense bodies of the platelets (41, 46). Strong inducers such as thrombin, collagen and latex particles trigger both release I and release II, whereas weaker inducers such as ADP and adrenaline are only able to cause release I (See Table 1). It is thought that once the platelet function has reached the stage where release occurs, the dense bodies are emptied. Only then, if the initial

stimulus has been sufficient, are the contents of the alpha granules released (47).

The exact process that triggers the release mechanism remains unclear (31, 46, 48-52) however, it is now recognised that the prostaglandin system plays a central role in initiating the release I reaction (53, 55).

Prostaglandins were first discovered more than 40 years ago by Goldblatt (56) and the pure forms were isolated and identified in 1960 by Bergstron and Sjovall (57). They are biosynthesised from polyunsaturated fatty acids (58, 59), primarily by arachidonic acid, which is a major component of membrane phospholipids (60). This is released from cell membranes by the action of phospholipase A_2 (61). The actual method by which this enzyme is activated is not fully understood (60,62) but it is believed that simple mechanical stimulation can result in the generation of prostaglandins from the platelets, and the lungs and the spleen (63-65). The diversity of the biological actions of prostaglandins became apparent in the studies of their effect on the cardiovascular and respiratory systems (66) and reviews (60, 66, 67, 68) confirm the complexity of their functions and structures. This thesis, however, describes the activity and aggregation of platelets and therefore only two products of prostaglandin synthesis are discussed and described. These are thromboxane A_2, a strong inducer of platelet aggregation, and prostacyclin, an inhibitor of aggregation.

Figure 4 describes the complete metabolic-pathway of prostaglandin synthesis. Both thromboxane A_2 and prostacyclin are derived from arachidonic acid and once this has been released by the action of phospholipase A_2, it is metabolised by two enzymes (69). The first is lipoxygenase which forms an unstable compound 12-hydroperoxy-arachidonic acid (HPETE) and its stable end product 12-hydroxy-arachidonic acid (HETE). The other enzyme is cyclo-oxygenase which forms prostaglandin endoperoxide PGG_2. This can be transformed enzymatically into two unstable products with potent biological activity called thromboxane A_2 (TXA_2) and prostacyclin (PCl_2).

Fig. 4 showing metabolic pathway of arachidonic acid.

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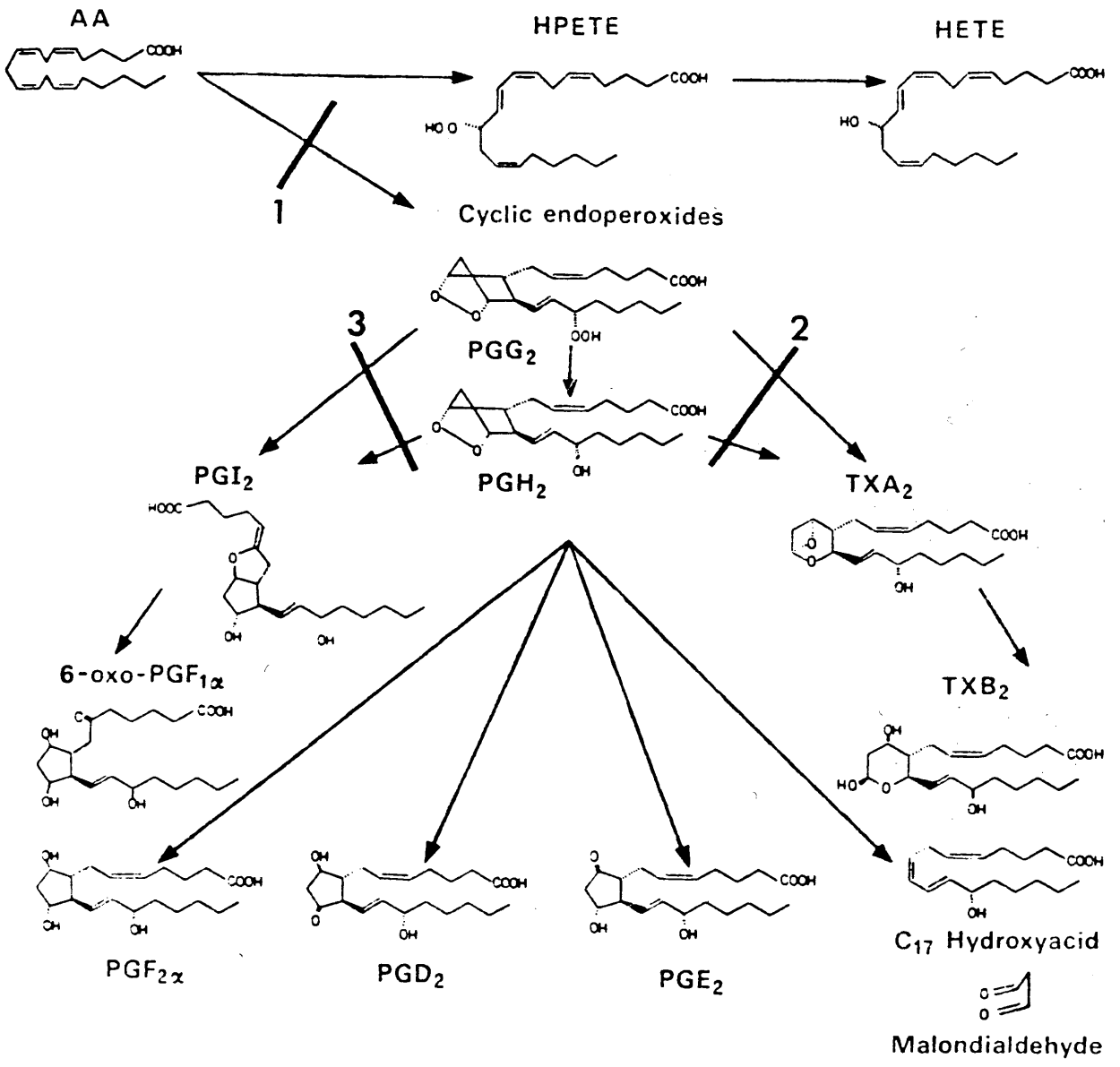
From Moncada, S. & Vane, J. R. (1978), p.130, (69).

Metabolic pathway of arachidonic acid



Abbreviations:

AA: arachidonic acid
HETE: 12-hydroxyarachidonic acid
HPETE: 12-hydroxyperoxyarachidonic acid
PGI_2: prostacyclin
TXA_2: thromboxane A_2
TXB_2: thromboxane B_2

The sites where cyclo-oxygenase inhibitors (aspirin-like drugs), thromboxane synthetase inhibitors and prostacyclin synthetase inhibitors exert their action are indicated by the numerals 1, 2 and 3, respectively

In platelets, the prostaglandin endoperoxides exert their effect through the conversion of the arachidonic acid into thromboxane A_2 (70). This is an unstable substance (half life 30 secs.) which is converted to thromboxane B_2 which plays no active role in platelet aggregation (69). Thromboxane A_2 is a strong inducer of platelet aggregation (71), which it achieves by triggering the release I reaction (1). This prostaglandin was so named because it was identified as the metabolite of arachidonic acid in the thrombocyte (platelet). The metabolic-pathway of arachidonic acid in the platelet is summarised in Fig. 5.

In constrast to the thromboxane A_2 metabolism in the platelets, prostacyclin (PGI_2) is the major product of arachidonic acid metabolism in all vascular tissue (69, 72-76). It is an inhibitor of platelet aggregation and its role will be discussed in greater detail in the subsequent section describing coronary heart disease (1.1.2, page 10).

In summary, the "fluffy coat" of the peripheral zone of the platelets plays an important role in the adhesion and aggregation of blood platelets. The processes involved in these reactions have been detailed in this section.

Located beneath this "fluffy coat" is the platelet membrane (1). Platelet specific proteins are arranged asymmetrically within the membrane and it has been demonstrated that only a few of the membrane proteins, including the three major glycoproteins are exposed to the outer aspect of the membrane (77-78). Immunological investigations have demonstrated that these glycoproteins play a major role in the adhesion of the platelets to the vessel wall (79-81).

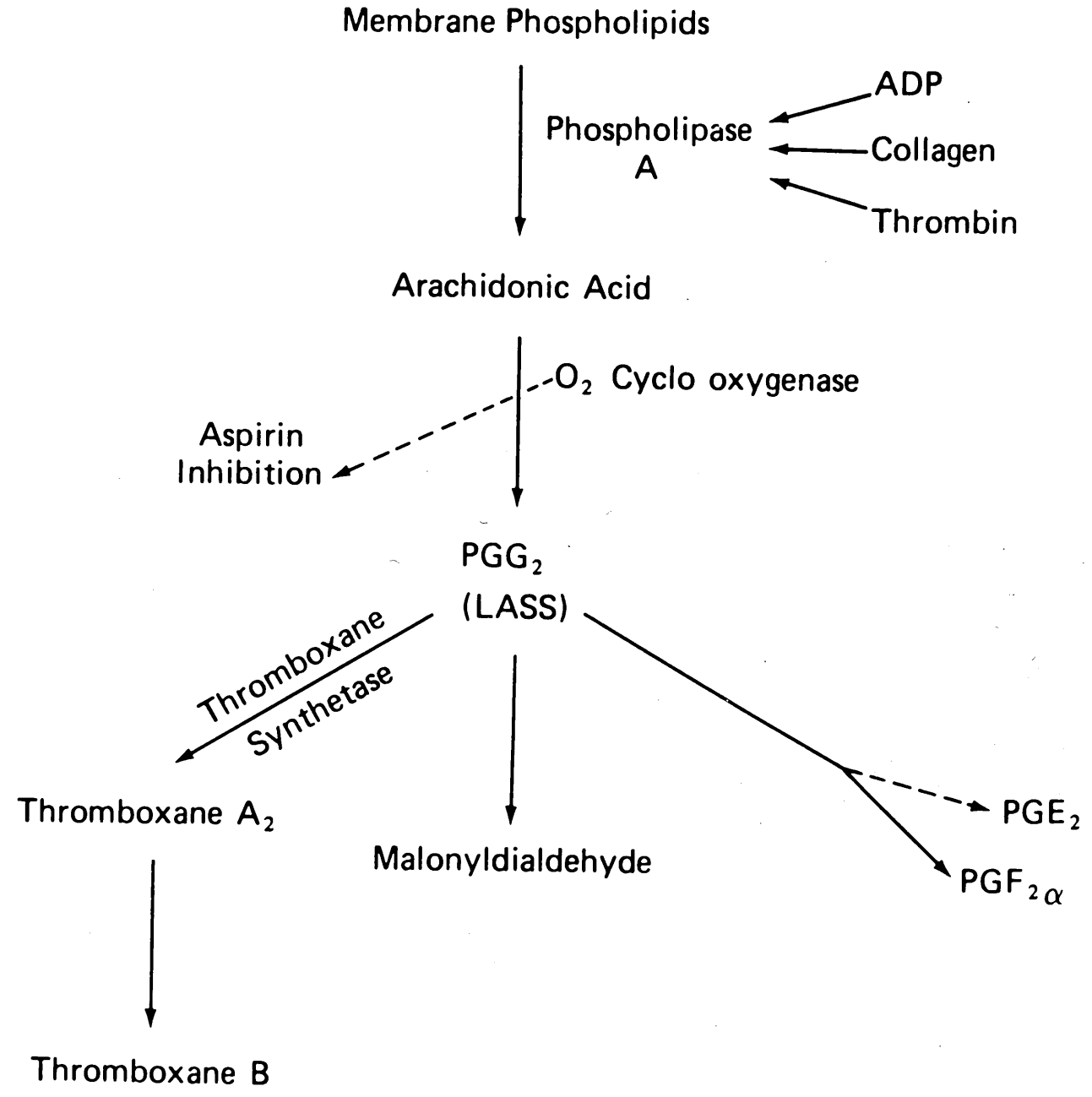
Another aspect of the platelet membrane is the network of channels that burrow through the platelet cytoplasm (1). This system, which has been called the surface connecting or open channel system, enlarges the total surface area of the platelet membrane and is thought to play a role in the rapid

Fig. 5 showing the role of prostaglandins in the platelet-release reaction and aggregation.

The arachidonic acid is released from the platelet phospholipids by the action of phospholipase A. It is subsequently metabolised by the enzyme cyclo oxygenase which forms the prostaglandin endoperoxide PGG_2 or labile aggregation stimulating substance (LASS) which is the precursor of thromboxane A_2.

Also included is the action of aspirin on the platelet. This drug inhibits the cyclo oxygenase thus preventing the formation of thromboxane A_2 and hence the release of the dense granules. This will be discussed in Chapter 2.

From Triplett, D. A. et. al. (1978), p.10 (1).


Membrane Phospholipids
ADP
Phospholipase A
Collagen
Thrombin
Arachidonic Acid
O_2 Cyclo oxygenase
Aspirin Inhibition
PGG_2 (LASS)
Thromboxane Synthetase
Thromboxane A_2
Malonyldialdehyde
PGE_2
$PGF_{2\alpha}$
Thromboxane B

extrusion of granule-bound secretory products during the release reaction (82).

(b) The sol-gel zone contains at least three systems of fibres which are present in the matrix of the platelets. These include the submembrane filaments, microtubules and microfilaments. The most prominent of these systems is the circumferential band of microtubules (13). It appears that the main function of these fibres is to maintain the discoid shape of the cells (1). During aggregation they migrate to the centre of the cell encircling the cluster of platelet granules (83-87). Their exact function in the release action is not well understood but the experimental removal of these tubules results in the impairment of the second or irreversible stage of aggregation (1). There is evidence to show that the microtubules regulate the release of the platelet contractile protein called actin (88).

(c) Two types of granules have been identified within the organelle zone of the platelets. The dense granules are homogenous, containing ATP, ADP, pyrophosphate, a divalent metal and permeability increasing factor serotonin (89). The number of dense bodies per platelet can vary from two to ten (1). The other granules located in the platelet cytoplasm are referred to as the alpha granules. These are heterogenous and only contain protein, namely, coagulation factors (fibrinogen, factor V), platelet specific proteins (e.g. platelet factor 4), cationic proteins (growth factor, permeability factor, bacteriocidal factor, chemotactic factor) and also various glycoproteins (89). The role of these granules and the mechanism controlling their release have already been described in this section.

The cytoplasm also contains some mitochondria which contribute to the energy metabolism of the cell (1) and also glycogen particles which are usually concentrated in a single large mass (13).

The platelet is therefore a complex cell involved in the

coagulation process and vascular support. In vivo, it initiates this process by adhering to collagen and undergoing the release mechanism. This process is triggered by the formation of thromboxane A_2 and the released dense granules provide the ADP to induce further platelet aggregation and eventually, through the clotting system, the formation and contraction of the fibrin clot. During this process many platelet factors are released, including those which increase permeability.

1.1.2 Coronary heart disease. This is a term used to describe clinical heart disease due to lesions of the coronary arteries (90). The "Textbook of Anatomy and Physiology" (91) details the anatomy and diseases of the heart and its arteries. The book describes how the coronary arteries supply blood to the heart-wall and their exact location is illustrated in Fig. 6. The heart wall is composed mainly of "thick, contractile, middle layer of especially constructed and arranged cardiac muscle called myocardium" (Fig. 7). These myocardial cells receive blood through two small vessels, the left and right coronary arteries. When these arteries are diseased, the myocardial cells are deprived of oxygen (become ischaemic) and subsequently catabolic metabolism is too low to ensure the survival of the cells. This impairs the operation of the heart which results in malfunction of the muscle. Ultimately this can lead to the death of the patient. However, anastomoses exist between the small arterial vessels in the heart and it is possible for new ones to develop which can provide collateral circulation to ischaemic areas.

The most common disease of the coronary arteries is atherosclerosis and the consequent disturbance in the blood supply to the myocardium constitutes the essential cause of coronary heart disease (91). Friedberg (90) describes atherosclerosis as "hardening of the arteries" which is characterised by "intimal thickening due to lipid atheroma, fibrosis, calcification, necrosis and haemorrhage". These atheromatous plaques, or "tissue reaction to accumulated lipids", take

Fig. 6 showing the surface of the heart. The left and right coronary arteries are included in the illustration.

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From Davies, D. V. (Ed.) (1967). Grays Anatomy, 34th edition, p.643. Longmans, London.

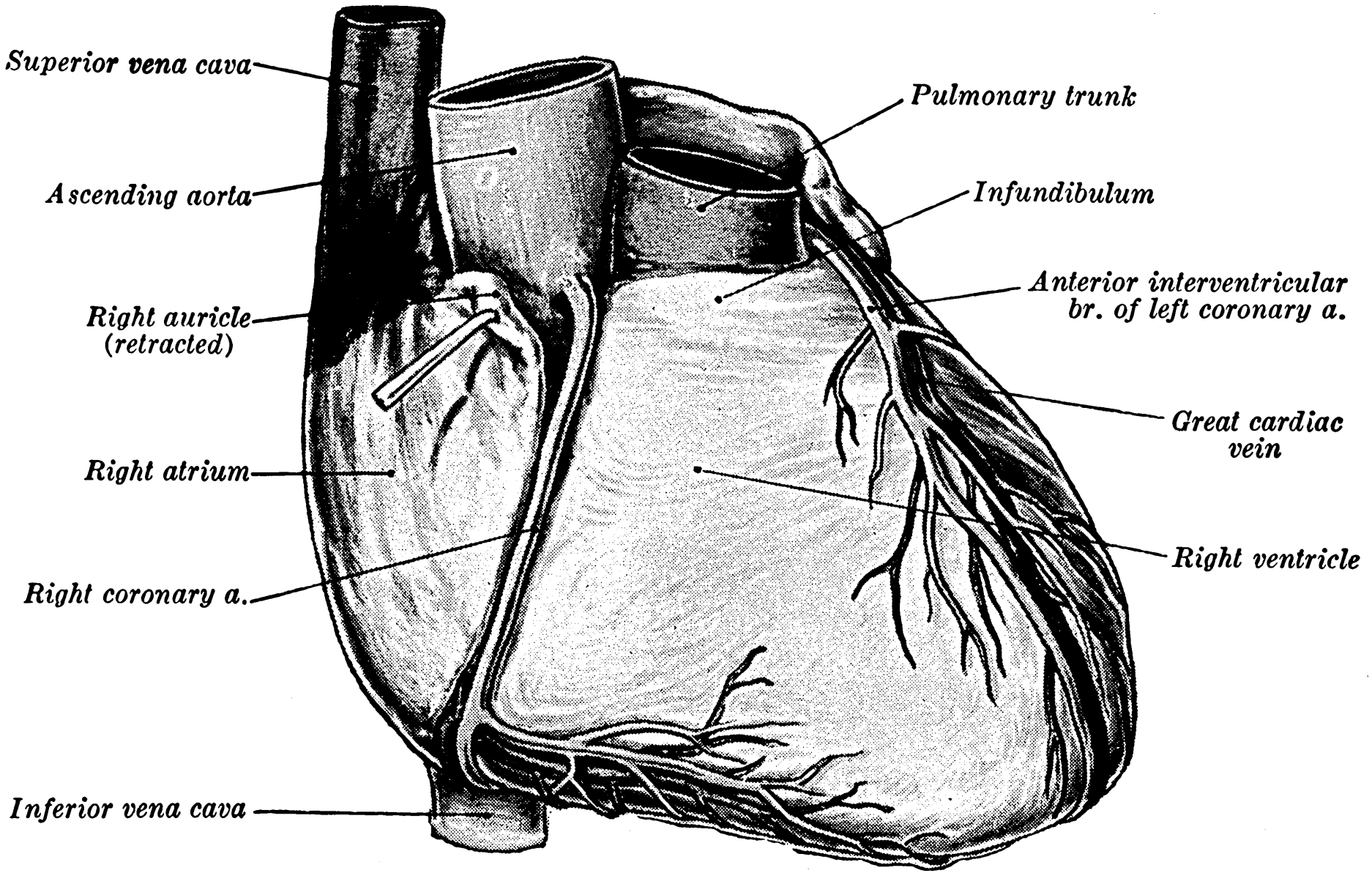

Superior vena cava
Ascending aorta
Right auricle (retracted)
Right atrium
Right coronary a.
Inferior vena cava
Pulmonary trunk
Infundibulum
Anterior interventricular br. of left coronary a.
Great cardiac vein
Right ventricle

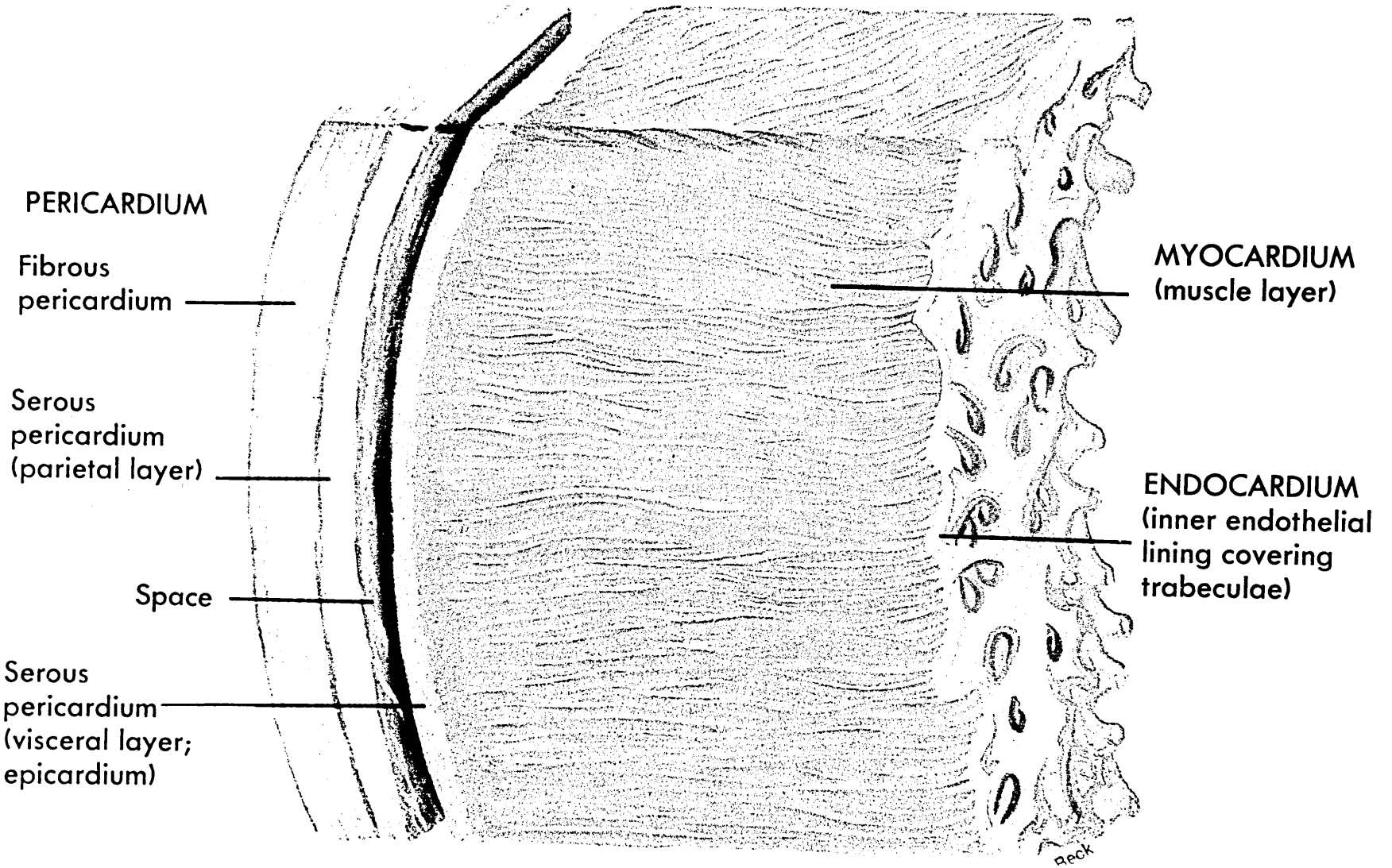
Fig. 7 showing a section of the heart wall. Included are the three main components:-

the outer pericardium (heart sac)

muscle layer (myocardium)

inner lining (endocardium composed of a layer of endothelial cells).

Reproduced with permission of the publishers and of the medical illustrator, Mr. Ernest Beck. From "Anatomy and Physiology", p.374 (91)


PERICARDIUM
Fibrous pericardium
Serous pericardium (parietal layer)
Space
Serous pericardium (visceral layer; epicardium)
MYOCARDIUM (muscle layer)
ENDOCARDIUM (inner endothelial lining covering trabeculae)
aeck

decades to form (90). The lesions which characterise the early development of atherosclerosis in humans are called fatty streaks (92). Different stages of development are recognisable (93). The ultimate stage is ulceration associated with the damage to the muscle and elastic tissue caused by the adherent thrombi and fibrous tissue (94).

Basically the theories describing the formation of these atheromatous plaques have hardly changed during the past hundred years (95). The thrombogenic hypothesis, reformulated by Duguid in 1949 (96) originated with the encrustation theory of von Rokitansky in 1844 (97). This theory involves the incorporation of thrombi into the arterial wall due to endothelial cells growing over the thrombus. Davies (95) suggests that this theory appears to be no more than a description of a process which may contribute to atheroma. Another hypothesis was originated by Virchow in 1862 (98). This relates atheroma to an inflammatory reaction in the arterial wall in the areas surrounding absorbed and trapped plasma constituents such as lipids and fibrinogen. These two hypotheses have been extensively reviewed by Spaet and Gaynor (99) and also Mustard and Peckham (100, 101). These reviews discuss and describe a definite relationship between thrombosis and atherosclerosis and it appears that the three important areas for consideration are:-

(a) the mechanism of thrombus formation.
(b) the relationship between this thrombosis and early changes in the vessel wall.
(c) the organisation of the mural thrombi and the development of atherosclerotic lesions.

The normal blood vessel is lined with endothelial cells (Fig. 8) which present an effective barrier between the blood and the underlying thrombogenic vascular components (99). If these endothelial cells are separated by tissue injury, the subendothelial tissues, such as basement membrane, are exposed (102). As previously described, the platelets can adhere to this membrane (2, 37) and also to collagen exposed by the separation of the endothelial cells (38, 103). The platelet

Fig. 8 showing the structure of artery wall. This varies with the size of the artery and its function. The drawings show (at different scales) the wall of the aorta (top) and of a small muscular artery (bottom).

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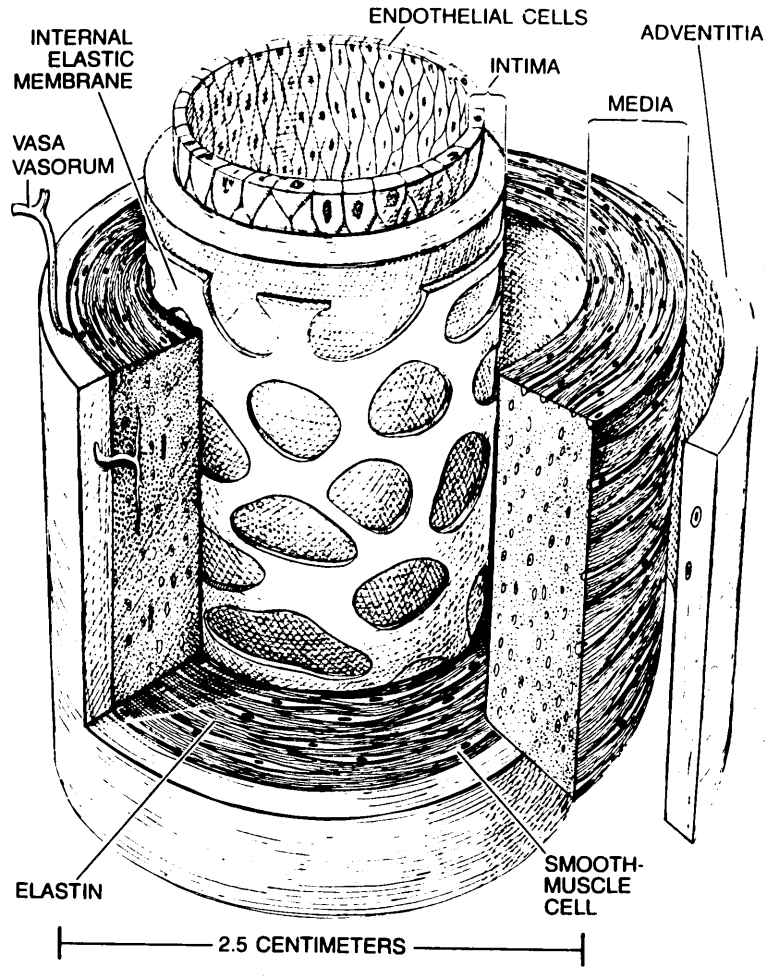
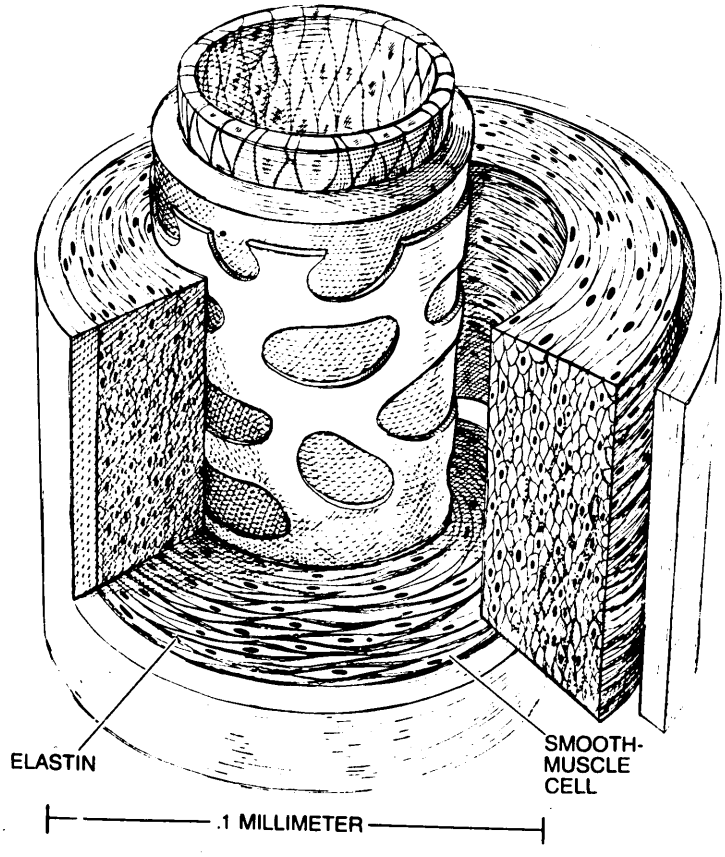

ENDOTHELIAL CELLS
INTERNAL ELASTIC MEMBRANE
INTIMA
ADVENTITIA
MEDIA
VASA VASORUM
ELASTIN
SMOOTH-MUSCLE CELL
2.5 CENTIMETERS


ELASTIN
SMOOTH-MUSCLE CELL
.1 MILLIMETER

aggregate formed is referred to as a thrombus (104). After the platelets adhere, the process is initiated resulting in the release of the dense bodies and therefore the ADP which cause aggregation to the platelets already adherent (10, 105). Further ADP for this release can be derived from injured cells in the vessel wall and also from red blood cells (106-109). The platelet rich thrombus formed is transformed into a fibrin mass and is subsequently organised into an intimal lesion which is rich in connective tissue (110, 111).

This incorporation of mural thrombi into the intima of arteries is a major and important factor in the build up of lesions that are involved in the development of atherosclerosis (112-114). In addition, during the formation of these thrombi the platelets release vaso active amines and these create areas of increased permeability. This results in further damage to the endothelium and also in the increased absorption of plasma constituents including lipids and fibrinogen into the arterial wall with the subsequent development of atheromatous plaques (101).

The hypotheses discussed above describe how the mural thrombi can be incorporated into the blood vessel wall and initiate a process which can eventually lead to the development of atheromatous tissue. During the last decade the factors that regulate the adhesion and aggregation of blood platelets <u>in vivo</u> have been extensively studied. One hypothesis, proposed by Moncada and Vane (69) suggests that the process is controlled by maintaining a critical balance between two metabolites of arachidonic acid - thromboxane A_2 and prostacyclin. As previously detailed in Section 1.1.1., page 7, thromboxane A_2 is the metabolite that mediates aggregation and release (71) and it has also been demonstrated that it is a potent vasoconstrictor (60, 115). Conversely, prostacyclin, which is synthesised in the vascular tisse (66), is a strong inhibitor of platelet aggregation and is also a vasodilator (69).

The mode of action of this process is uncertain but it appears that after the platelets adhere to the vessel wall the arachidonic acid in the surrounding vascular tissue is activated and the prostacyclin is produced (69). Controversy remains whether any cross activity exists between the arachidonic acid and the prostaglandin endoperoxides in the platelets and the vessel wall but it appears that after stimulation the prostacyclin is produced independently and without any influence from the platelet constituents (69). The endothelial cells in the vessel walls are the most active producers of prostacyclin (116, 117) and it is therefore abundant in the intima, but it progressively decreases in concentration from the intima to the adventitia. In contrast, the proaggregating elements increase from the subendothelium to the adventitia. These two opposing tendencies therefore render the endothelial lining antiaggregatory and the outer layer of the vessel wall thrombogenic (60, 118). This mechanism allows the platelets to adhere and repair any vascular damage, but the formation of the thrombus is controlled by the formed prostacyclin.

The mechanism which initiates prostacyclin synthesis in the vessel wall, after the adhesion of platelets has not been fully elucidated. However, the mode of action of its inhibitory effects on platelet aggregation has been established. Prostacyclin inhibits aggregation by stimulating adenyl cyclase which leads to an increase in the cyclic AMP (cAMP) levels in the platelets (119, 120). Cyclic AMP enhances Ca^{2+} sequestration (121) and the platelet bound Ca^{2+} is essential for the aggregation process, detailed in the previous section (1.1.1.), to proceed (122). A review by Luscher and his co-workers (122) describes how cytoplasmic Ca^{2+} plays an important role in the contractile activity and the release reaction of the platelets. Aggregation is inhibited by the increase in cAMP levels because thromboxane A_2 synthesis is dependent upon the availability of arachidonic acid which is cleared from phospholipids by the action of phospholipase A_2, an enzyme which requires Ca^{2+} for its activity. cAMP accelerates the removal of Ca^{2+} ions from the cytoplasm and accordingly decreases phospholipase activity. Therefore, the potent aggregating effect of the thromboxane A_2

in the platelet is regulated by the ability of the prostacyclin formed in the vessel wall to increase the cAMP levels in the cells. This process is illustrated in Fig. 9.

It is therefore important that the body maintains this critical balance between the levels of thromboxane A_2 and prostacyclin and any factor that inhibits the formation of the latter can result in a thrombotic state. For example, inhibition of the prostacyclin formation by lipid peroxides can lead to a condition in which platelet aggregation is increased and this could play a role in the development of atherosclerosis (60). It has been shown that lipid peroxidation takes place in plasma as a nonenzymic reaction (123) and is also known to occur in certain pathological conditions (124). Therefore the lipid peroxides present in these conditions could unbalance the system in favour of the thromboxane A_2 and predispose to thrombus formation (60). It has also been reported (125) that human atherosclerotic tissue does not produce prostacyclin, whereas tissue obtained from a nearby normal vessel does.

Evidence therefore suggests that platelets can play a major role in the development of atheromatous tissue. This process can be initiated by the release of permeability factors from the platelets during the repair of vascular damage, thus encouraging the deposition of lipids and fibrinogen. The lipids deposited can also interfere with the thromboxane A_2/ prostacyclin balance which is thought to regulate the degree of thrombus formation <u>in vivo</u>.

One clinical manifestation of coronary heart disease is myocardial infarction (90). This signifies the necrosis or death of a portion of the heart muscle because of an interruption or curtailment in the blood supply to the myocardium. Myocardial infarction can be diagnosed using clinical and laboratory tests and in this thesis only subjects fulfilling certain strict criteria, described later in the text, are included as patients with confirmed coronary heart disease.

Fig. 9 showing the role of adenosine 3',5'-cyclic monophosphate (cyclic AMP) in the inhibition of platelet aggregation.

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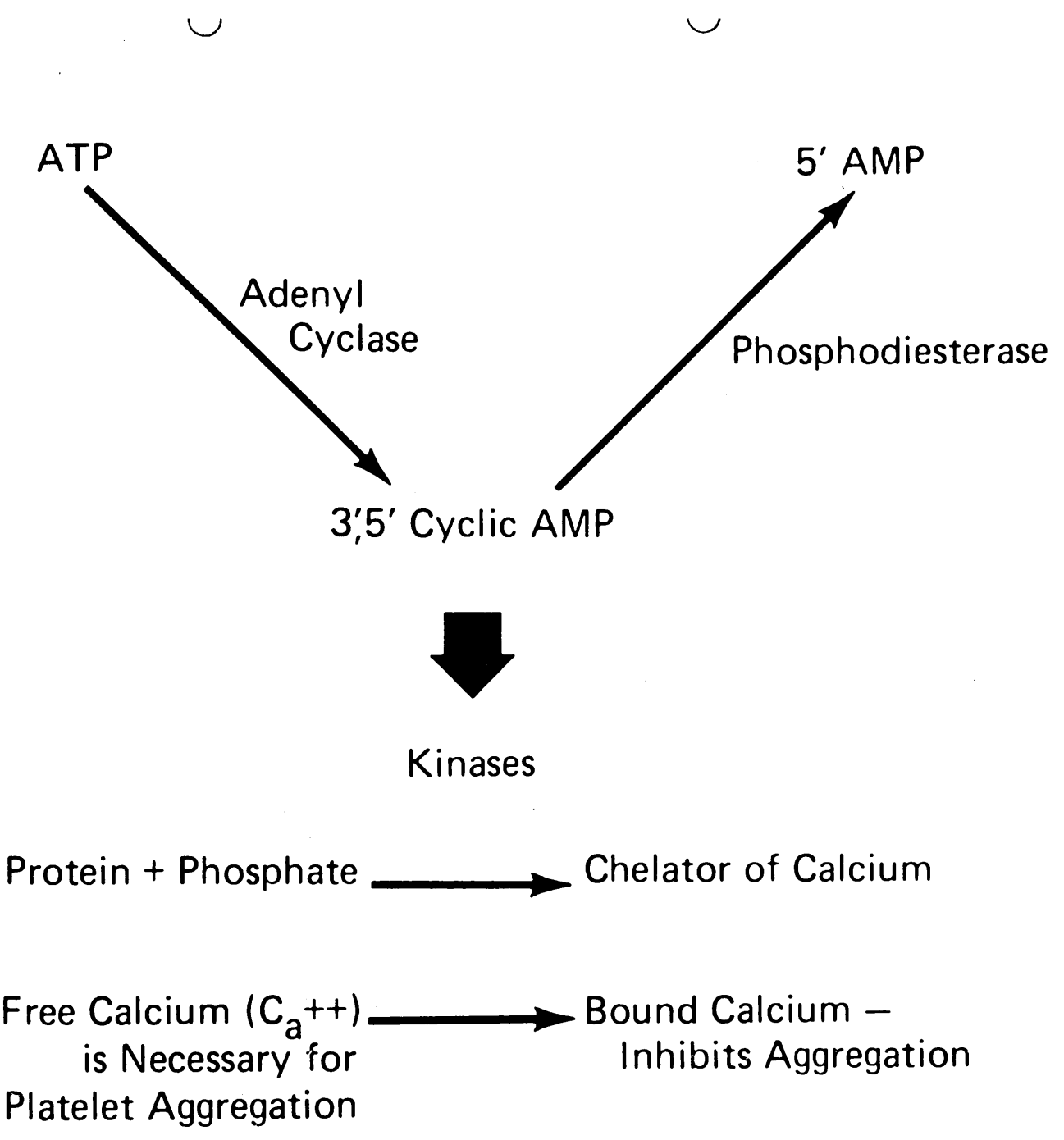
ATP
5′ AMP
Adenyl
Cyclase
Phosphodiesterase
3′,5′ Cyclic AMP
Kinases
Protein + Phosphate
Chelator of Calcium
Free Calcium (C_a++)
is Necessary for
Platelet Aggregation
Bound Calcium –
Inhibits Aggregation

1.1.3 The role of "active" platelets in coronary heart disease. The previous section discusses the reported role of platelets in the formation of the thrombus and in the subsequent development of atheromatous plaques. The platelet work described in this thesis is concentrated on the observations that coronary heart disease is one condition where an increased percentage of "active" platelets are present in the patient's circulation (126). Their presence has also been confirmed by the demonstration of an increased rate of platelet turnover in CHD (1, 127-130).

The presence of these "active" platelets could be related to the effect of the atheromatous lesions and subsequent ulceration, however, some workers believe (100, 105) that platelets may initiate the vascular damage and accelerate the formation of the subsequent atheromatous plaques. An attempt was made to avoid testing hospital-based patients in the acute phase of the disease. There is a possibility that changes found in such patients may be the result of the infarction. They can be due to, for example, ulceration of the endocardium near to the necrotic myocardium, or due to the patient being in a state of shock or to medication administered in the acute phase. In an attempt to elucidate their role, an experimental programme was devised which investigated the platelet activity in "long-term" confirmed coronary heart disease patients and in a large number of normal control subjects. The assumption was made that any damage present in the acute phase of the disease had healed in these patients and any differences found in case-control studies could be related to atherosclerosis. All the confirmed MI patients investigated in this thesis had suffered the infarct at least twelve months prior to the test.

In order to describe the role and relevance of an increased percentage of these platelets in the circulation, the term "active" platelets must be explained and discussed.

The normal function of platelets has already been

detailed. The mechanism involved in maintaining the platelet metabolism in order to perform these roles should also be considered. The reviews by Wintrobe (10) and others (1, 13) describe how the energy metabolism of the platelet resembles that of the skeletal muscles. Both involve active glycolysis and the synthesis and utilisation of large amounts of glycogen. The mediator of intracellular energy utilisation is an actomyosin-like adenosine tri-phosphate and the major energy source for the platelet is glucose which is rapidly taken up from the plasma. The glycolytic pathway with its regulatory enzymes, the tricarboxylic acid cycle, the pentose-phosphate pathway and the NAD-NADH system are all active in the platelet (131). Approximately 90% of free platelet nucleotide consists of adenine nucleotides and are synthesised from both adenanine and adenosine. Platelet nucleotides are partitioned into at least two different pools (45, 132). The metabolic pool is utilised for the maintenance of the various energy consuming cell functions. This pool mainly consists of ATP in contrast to the storage pool which contains both ADP and ATP. The nucleotides in this latter pool are metabolically inactive and are extruded from the cell during the release reaction. The ATP that has been used to provide the energy requirements of the cell is thought to be stored in a third compartment in equilibrium with the metabolic pool (45). ATP can also be converted into cyclic AMP which plays an important role in regulating platelet function. In the normal platelet a constant and critical level of cAMP is maintained by the enzyme adenyl cyclase and only after platelet stimulation is the level changed. It appears that normal platelet activity is predetermined, the platelets being furnished with a level of nucleotides to maintain their function for their 9 to 11 days of life (13, 133). The activity of the platelets also decreases steadily during this period terminating in the removal of the effete cell by the reticulo-endothelial system. Therefore, when platelets first enter the circulation, they demonstrate a higher degree of activity than that observed towards the end of their period in the blood stream when their energy providing mechanisms have expired. It has been shown that the "young" platelets entering the blood stream are the most metabolically active (30, 134-137). These cells are "larger and more dense.

They adhere more readily to collagen, aggregate more rapidly with ADP and show increased aggregation with stimuli such as epinephrine , thrombin and collagen" (137). These larger platelets release a greater amount of ADP during the release process (30, 134) in addition to an increased level of permeability increasing factors at the site of aggregation. Karpatkin and his co-workers (30, 134) list the properties of this population of platelets in comparison with the older less active cells. Shulman et. al. (22) suggest that only this newly formed population of platelets, capable of synthesising proteins, are haemostatically effective. They comprise of 20-25% of the total circulating platelet population and it appears that this level of activity is maintained for approximately 2 days. This thesis examines the importance and significance of the presence of an increased percentage of these "young" platelets and in the text these cells are referred to as "active" platelets.

It is known that coronary heart disease is one condition where platelet turnover is increased with no effect on the total platelet count (1). This has been confirmed by the demonstration of an increased percentage of "active" platelets in this condition (126). This thesis examines and explores the possibility that the process of vascular repair and the regulatory balance between thromboxane A_2 and prostacyclin function efficiently when the population of platelets consists of the normal 20-25% of "active" cells. It is hypothesised that a population of platelets with a higher number of "active" cells can unbalance the protective mechanisms resulting in the acceleration of the process which leads to the development of atheromatous plaques.

The effect of the "active" platelets on the normal process of vascular repair must be considered. These "active cells" "preferentially adhere to collagen" (138) and therefore more platelets than usual would be stimulated to respond to the vascular repair mechanism. In addition, each individual cell would release more ADP (30, 134) thus encouraging the formation of a larger and sturdier thrombus. An increased amount of permeability increasing factors would also be available and on

release from these platelets would encourage the deposition of plasma constituents such as lipids and fibrinogen. These large "active" platelets have more phospholipid content than the less active cells and it has been demonstrated that platelets from CHD patients show a greater response to arachidonic acid stimulation when compared with normal control subjects (139). These cells must therefore produce more thromboxane A_2 thus encouraging platelet aggregation and release on the damaged vessel wall. However, the prostacyclin response would not demonstrate the equivalent exaggerated stimulation, thus decreasing the antiaggregatory effect and encouraging thrombus formation and its subsequent organisation into the vessel wall.

The theories that platelets play an important role in the development of atherosclerosis has been reviewed by Born (109) who believes that it is questionable whether platelets can remain on the arterial wall for a sufficient period of time to initiate the process referred to in the previous section. However, it has been established that platelets repair and are incorporated into the damaged vessel wall (32-35). Wook and Carstairs (140) describe how platelet material has been isolated in atheromatous tissue. Ross and his co-workers (141) suggest that platelets are essential for the development of intimal hyperplasia seen in atherosclerotic plaques because they release a mitogenic factor that stimulates the proliferation of the smooth muscle. Evidence therefore suggests that platelets play a definite role in the development of atheromatous lesions. It could be speculated that a thrombus formed from a normal population of platelets could conceivably be removed from the vessel wall by the mechanical effect of the blood flow. However, the presence of an increased percentage of "active" platelets in a patient's circulation could exaggerate the response to vascular damage, form a sturdier thrombus which could be organised into the vessel wall and result in the process already detailed.

In this thesis the role of an increased percentage of "active" platelets in the circulation of confirmed coronary heart disease patients has been approached in two ways.

The first approach accepts that the increased percentage of "active" platelets is secondary to the atheromatous tissue and the subsequent ulceration. Coronary heart disease is a condition where the atheromatous deposits are created over a long period of time while the individual shows no evidence whatsoever of the disease (142). The presence of an increased percentage of "active" platelets in apparently healthy subjects could therefore be the only indication that such a process was occurring. A test was developed, suitable for use on a large number of subjects, capable of reflecting the presence of an increased number of these "active" platelets. The presence of an increased number of these cells and therefore a greater platelet turnover than normal could be the only recognisable factor in detecting individuals who are undergoing a process which ultimately terminates in clinical symptoms of coronary heart disease.

Most of the workers who investigated the phenomenon of increased platelet activity in CHD based their observations on hospitalised patients in the acute phase of the disease. Therefore, before investigating platelet activity in normal subjects, the activity in "long-term" patients should be established. Confirmed coronary heart disease patients included in the experiments described in this thesis have suffered from the myocardial infarction at least twelve months prior to testing. The significance of this time factor has been discussed on page 15. The result of such investigations should ascertain whether a higher than normal percentage of "active" platelets remain in the circulation of confirmed CHD patients.

The tests available for the detection of this population of platelets are time-consuming and some require expensive and specialised equipment (126, 128-130). It was therefore essential to develop a test, suitable for use in a clinical laboratory, capable of detecting a greater than normal percentage of "active" platelets in a large number of individual subjects. The test developed and the results obtained are detailed in Chapter 2.

The second approach to the presence of an increased percentage of "active" platelets explores the hypothesis that these platelets can play a primary and causative role in initiating the process that cumulates in the development of the atheromatous plaques. This hypothesis is based on the knowledge that an increased turnover of platelets can result from many factors to which every individual is susceptible and include viraemia, bacteraemia and antigen-antibody complexes (137, 143-145). Certain IgG class antibodies can sensitise the platelets resulting in their removal from the circulation (146-153). This leads to an increased turnover of the platelet population and therefore a higher percentage than normal of "active" platelets in the blood.

It has been demonstrated that vascular damage can be caused by the direct action of platelets. Mustard and Packham (100, 101) and others (96, 112-114, 154) describe how "there is considerable evidence that the organisation and incorporation of mural thrombi into the intima of arteries is the major factor in the build-up of stenotic lesions that are involved in the development of complications of atherosclerosis". One contributory factor is the rate and pattern of the blood flow (blood rheology) (100, 101, 155, 156). The mechanical effect related to the pattern of the blood-flow could be the initiating factor in the characteristic localisation of the early vascular changes. Oka (157) in his review of the current literature on this aspect of CHD states that any theory for the formation and growth of atherosclerotic plaques should take into account "the flow characteristics of the blood". He describes the current hypotheses which are classified as pressure, wall shear stress, turbulence and flow separation related. These hypotheses describe how the blood-flow pattern can initiate the vascular damage (158, 159) and how the turbulent velocity fluctuations cause platelets to adhere to the vessel wall (160). Blood-flow patterns can also initiate the release of factors from the platelets and also the vessel wall which causes increased permeability at the site of reaction (101, 161). The direct action of platelets on the endothelium in these areas of disturbed blood flow is another contributory

factor which can result in vascular damage (100, 101, 160). Thrombi formed in these areas can also fragment and shower the arterial wall with platelet fragments, thus causing further endothelial damage (101). Intravascular platelet aggregates can also be directly induced by stimuli such as endotoxins, viruses, bacteria and antigen-antibody complexes and these thrombi can be directly responsible for vessel injury (105, 162). It is to be expected that the enhanced effect and damage which is caused in the circulation when the platelet population consists of a greater than normal number of young "active" platelets will exaggerate the processes referred to above.

The study of the platelet population in "long term", confirmed coronary heart disease patients and a large number of apparently healthy normal subjects should aid in elucidating the role of the "active" platelets in this condition. The results of the experimental programme designed and the conclusions drawn are reported and discussed in the text.

1.1.4 The immunological theory of coronary heart disease. The immunological theory originated from the observation by Davies (163-165) that the negative charge on the erythrocyte suspended in plasma was reduced in the blood of patients with confirmed coronary heart disease. It was demonstrated that this abnormality was due to a factor present in the plasma of these patients. Further experiments demonstrated that the platelets and chylomicra showed a similar pattern in patients with this condition (166-168). The influence of the plasma in modifying the electrical charge on particulate matter was termed by Davies (169) as "plasma surface activity". Work continued to identify and isolate the factors present in the plasma responsible for reducing the negative charge on the erythrocytes. It was shown by Davies and Clark (170) that the blood of confirmed coronary heart disease patients not only showed a reduced electrophoretic mobility but the γ G-globulin (IgG) level in these patients was also elevated. It was postulated (142) that these

immunoglobulins coated the cells, including the platelets, thus making them more adhesive. In addition, the authors suggest that the increased IgG levels in confirmed CHD patients reduce the plasma surface activity and point to the possibility of an immunological factor being involved in the disease. The higher level of IgG was observed in these patients over a period of time confirming that the findings were not secondary to the infarction. The raised immunoglobulin level in patients with confirmed CHD has subsequently been confirmed by other workers (171).

It was considered by Davies (142) that the aetiological factors that could be involved in this new approach could conceivably be endogenous or exogenous or both. As previously stated, atheromatous deposits and coronary artery disease are created over a period of time while the individual may show no evidence whatsoever of the disease. Davies (142) believed that the lack of any clinical symptoms suggested that any search for the antigens should initially be concerned with the exogenous factors. Relevant to this approach was the published evidence of the intestinal absorption of intact dietary proteins with the creation of specific antibodies to these antigens. This factor is discussed in detail in Chapter 8. Recorded evidence (172-177) showed that a proportion of the apparently healthy population appeared to produce antibodies, sometimes to a high titre, to various foods. The food most commonly implicated by these workers was milk.

This approach to coronary heart disease was pursued with a series of tests, initially concentrating on the levels of antibodies to milk protein in confirmed coronary heart disease patients and normal control subjects. The results of these investigations were reported by Davies et. al. (178) who concluded that antibody titres to whole dried milk were higher in patients with confirmed CHD than in the normal control subjects tested. The possible harmful effects of these antibodies have been documented in respect of sudden death in babies (179) and in ulcerative colitis (177) but no relationship had previously been reported relating the presence of

bovine milk antibody to coronary heart disease.

Circumstantial evidence for the involvement of milk protein in the aetiology of atherosclerosis has been discussed by Davies (95). He describes the findings of Osborn (180), who studied coronary arteries of 109 young individuals and found where there had been no breast feeding the coronary arteries were mainly abnormal. A similar conclusion was also drawn by Turner (181) and Osborn (182). Muir (183) refers to the work of Annand (184) who demonstrated that an increase in the incidence of ischaemic heart disease occurred soon after the introduction of Holder pasteurisation process. Annand (185) and Segall (186) correlated the consumption of milk proteins to the incidence of coronary heart disease and referred to Finland which has the highest coronary mortality rate and the highest per capita intake of total milk. Recently (187), Seely reported that a "direct and reasonably accurate correlation has been found between coronary heart disease mortality rates and the consumption of unfermented milk proteins". This correlation is illustrated in Fig. 10.

The hypothesis that the development of atherosclerosis was the result of an immunological disorder was first published by Davies in 1969 (142). Evidence in support of this immunological approach has since been collected and reviewed (95, 188). These publications describe how the immunological hypothesis embodies the two long-standing theories of coronary heart disease already described in section 1.1.2. The direct action of immune antigen/antibody complexes can initiate the vascular damage and the subsequent formation of the thrombus (189). In addition, antibodies and complement have a toxic action on the vascular endothelial cells (190). Mast cells present in the arterial wall are activated by antigen/antibody complexes resulting in the release of permeability-increasing factors (191). Davies (142) suggests that the excess lipids in atheromatous arterial lesions may be due to excessive filtration resulting directly from this increased permeability. Most of the factors that cause increased permeability results in the

Fig. 10 demonstrating the correlation between coronary heart disease mortality rate and the consumption of unfermented milk proteins.

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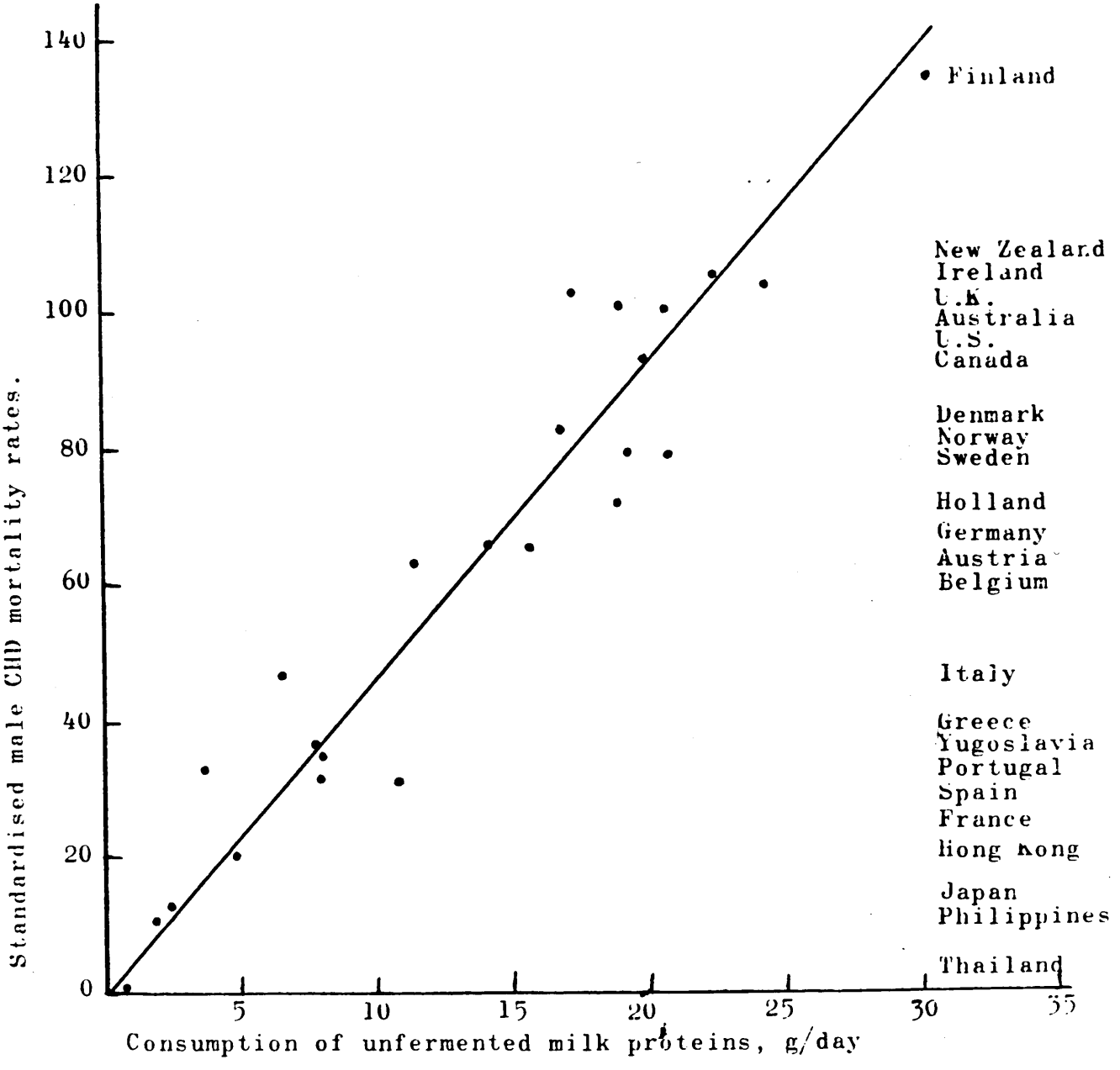

Standardised male CHD mortality rates.
140
120
100
80
60
40
20
0
5
10
15
20
25
30
35
Consumption of unfermented milk proteins, g/day
Finland
New Zealand
Ireland
U.K.
Australia
U.S.
Canada
Denmark
Norway
Sweden
Holland
Germany
Austria
Belgium
Italy
Greece
Yugoslavia
Portugal
Spain
France
Hong Kong
Japan
Philippines
Thailand

separation of the endothelium cells from each other thus exposing the subendothelial tissue such as basement membrane, thus encouraging platelet adhesion (102). Serum sickness experiments in animals have demonstrated that some antigen/antibody complexes are capable of causing vascular damage by being deposited directly in the arterial wall (188). As previously described, the development of an immunological disturbance leads to platelet aggregation which can have several different results. Briefly, the platelet aggregates formed can cause vessel injury (96, 100, 101, 112, 114, 154), permeability increasing factors are released by platelets (89), certain immune antigen/antibody complexes and IgG antibodies not only cause platelets to aggregate, but they also result in the removal of the cells by the reticulo endothelial system, thus causing an increased platelet turnover (137, 143-153). The importance of an increased percentage of "active" platelets has already been discussed in section 1.1.3.

More recent findings, as the result of heart transplantation, offer further evidence that immunological factors play a major role in the development of atheroma. Gross atherosclerosis developed in the coronary arteries of a transplanted heart, previously normal, in the 19 months before the death of the recipient (192). In a review similar findings have been described by Davies (193) who reported that "the main cause of death in long-term cardiac transplant patients is an accelerated form of coronary heart disease (atherosclerosis)". (194).

In summary, the aims of this thesis are:-

1. To attempt to elucidate the role of "active" platelets in coronary heart disease. To achieve this, a technique was developed capable of testing a large number of "long term" confirmed CHD patients and apparently normal healthy control subjects. This section of the work was approached in two ways:-

   (a) The theory that the presence of an increased

percentage of "active" platelets is secondary to atheromatous tissue was provisionally accepted and a technique developed of sufficient sensitivity to detect these cells in the "long-term" confirmed CHD patients. The value of the test as a predictor of the presence of CHD in the apparently normal healthy subject is also examined and discussed.

(b) The data obtained was examined to ascertain if any evidence existed to support the hypothesis that these "active" platelets could be one important factor in the initiation of the process which terminates in clinical coronary heart disease.

2. To confirm the observation that a relationship exists between bovine milk antibody and coronary heart disease.

3. To isolate and identify this antibody, report the findings and to relate any relationship to metabolically "active" platelets.

CHAPTER 2

ADP "DISAGGREGATION RATIO"

2.1 Introduction

The tests currently available for measuring the rate of platelet turnover and hence the number of "active" platelets are unsuitable for the investigation of large numbers of subjects. Most of the studies utilise radioactive isotope techniques, the main radio-nuclides used being ^{51}Cr and ^{32}P. These techniques are time-consuming and involve the manipulation of platelets *in vitro* during the labelling procedure (195). The equipment used is also specialised and expensive. Shatz and Riddle (126) used electron microscopy studies to demonstrate an increased percentage of "active" platelets in the circulation of confirmed myocardial infarction patients. Another non-isotope method was described by Stewart and his co-workers (196). This test was based on the measurement of malondialdehyde (MDA), an end-product of platelet lipid peroxidation. This process has already been described on page 7 and Table 5 illustrates the formation of thromboxane A_2 and also malondialdehyde. De Haas and his co-workers (195) describe how lipid peroxidation is stimulated by adding collagen, thrombin, l-epinephrine, ADP or N-ethylmaleionide to prepared platelet suspensions. The process is blocked irreversibly by acetylsalicylic acid (ASA) and this inhibition persists for the remaining life span of the circulating platelet . ASA prevents the formation of MDA by interfering with the enzyme cyclo oxygenase. This action is also illustrated in Fig. 5. The principle of this test is that the emergence of new, unaffected platelets into the circulation is monitored by estimating the increase in MDA production in serial blood samples obtained over a period of 10 days. This constitutes a measure of platelet production time. De Haas *et. al.* (195) modified this method by using arachidonic acid to stimulate platelet lipid peroxidation. Again, this test, although not involving expensive and specialised equipment, requires monitoring of the patient for 10 consecutive days.

In order to test a large number of subjects on a survey basis, it was desirable to introduce a test that could be used in a hospital laboratory utilising the apparatus already available and with the minimum of inconvenience to the patients involved. The test developed was based on the aggregation patterns obtained when platelets are stimulated with ADP. The original aggregation method was devised by Born (197) and the principle of the test has been detailed on page 5. It is known that when a low concentration of ADP is added to a preparation of platelet-rich plasma (prp), the platelets aggregate, and because the level is insufficient to stimulate the formation of thromboxane A_2, the dense granules are not released and the platelets exhibit reversible aggregation. This is illustrated in Fig. 11. This phenomenon occurs up to a critical level of ADP (197), the addition of the aggregating agent at a higher concentration results in the formation of thromboxane A_2 in sufficient quantity to cause the release of the dense granules. When this occurs the prp specimen tested exhibits irreversible aggregation (See Fig. 11).

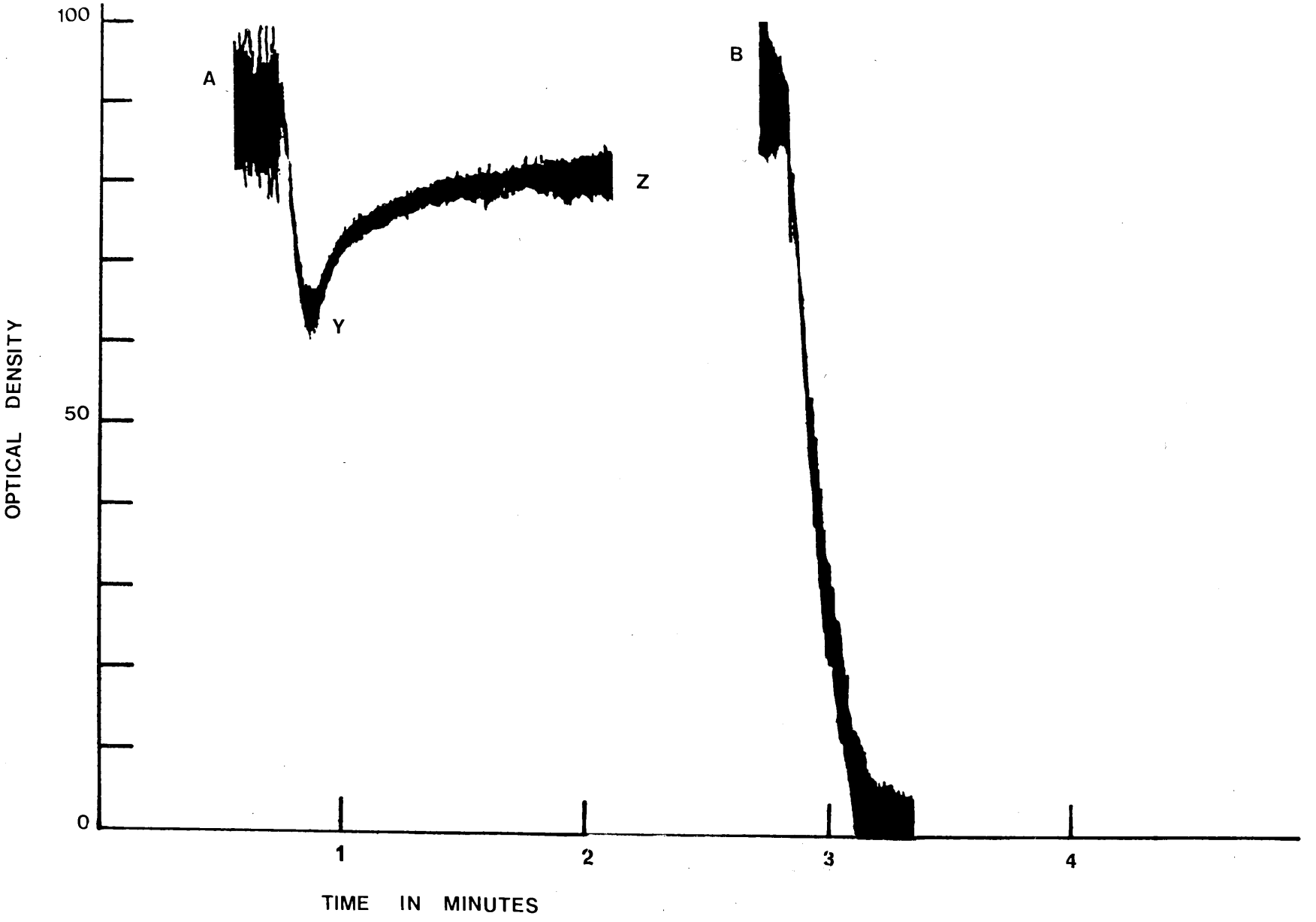
A test was developed utilising the above observations. When the highest possible concentration of ADP that does not cause the release of the dense granules, is added to populations of platelets from normal donors, reversible aggregation should be observed. As previously mentioned on page 17, such populations of platelets should contain 20-25% "active" platelets. It is postulated that if the same ADP solution is added to samples of prp containing an increased percentage of "active" platelets, these, by virtue of their greater response to ADP (30, 134), will be stimulated to form thromboxane A_2 and hence the release of the dense granules and therefore irreversible aggregation. This degree of irreversible aggregation should reflect the activity of the platelet population tested in comparison to prp samples from normal donors. The highest possible concentration of ADP that allows reversible aggregation in normal samples of platelets has been termed "the critical dose" and the degree of irreversible aggregation obtained called the "disaggregation pattern".

Fig. 11 showing:-

"A"= reversible aggregation after the addition of 1 µg of ADP to 1 ml of platelet rich plasma (concentration of platelets 300 x $^{9}/_{1}$).

The OD change to "Y" represents the aggregation of the platelet population and the return to "Z" demonstrates reversible aggregation.

"B"= irreversible aggregation after the addition of 3 µg of ADP to the same population of platelets.


100
50
0
OPTICAL DENSITY
A
Y
Z
B
1
2
3
4
TIME IN MINUTES

It has already been described how aspirin (acetylsalicylic acid or ASA) prevents the release of the dense granules from the platelets by interfering with the enzyme cyclo oxygenase and the subsequent formation of thromboxane A_2. This phenomenon also interferes with the disaggregation patterns obtained with the "critical dose" of ADP by preventing the release of the dense granules from the "active" platelets. Patients receiving aspirin were excluded from surveys and therefore the prp specimens from every subject reported in this thesis were all tested against collagen in parallel to the "critical dose" of ADP. Collagen was used as it is the aggregating agent routinely used to demonstrate any interference on the aggregation of platelets by ASA (198). The effect of aspirin on the aggregation patterns obtained with collagen is illustrated in Fig. 12.

2.2 Materials and methods

The basis of the test developed was to obtain the "critical dose" of ADP. This was achieved by adding varying concentrations of ADP to preparations of prp samples from normal donors until normal disaggregation no longer occurred.

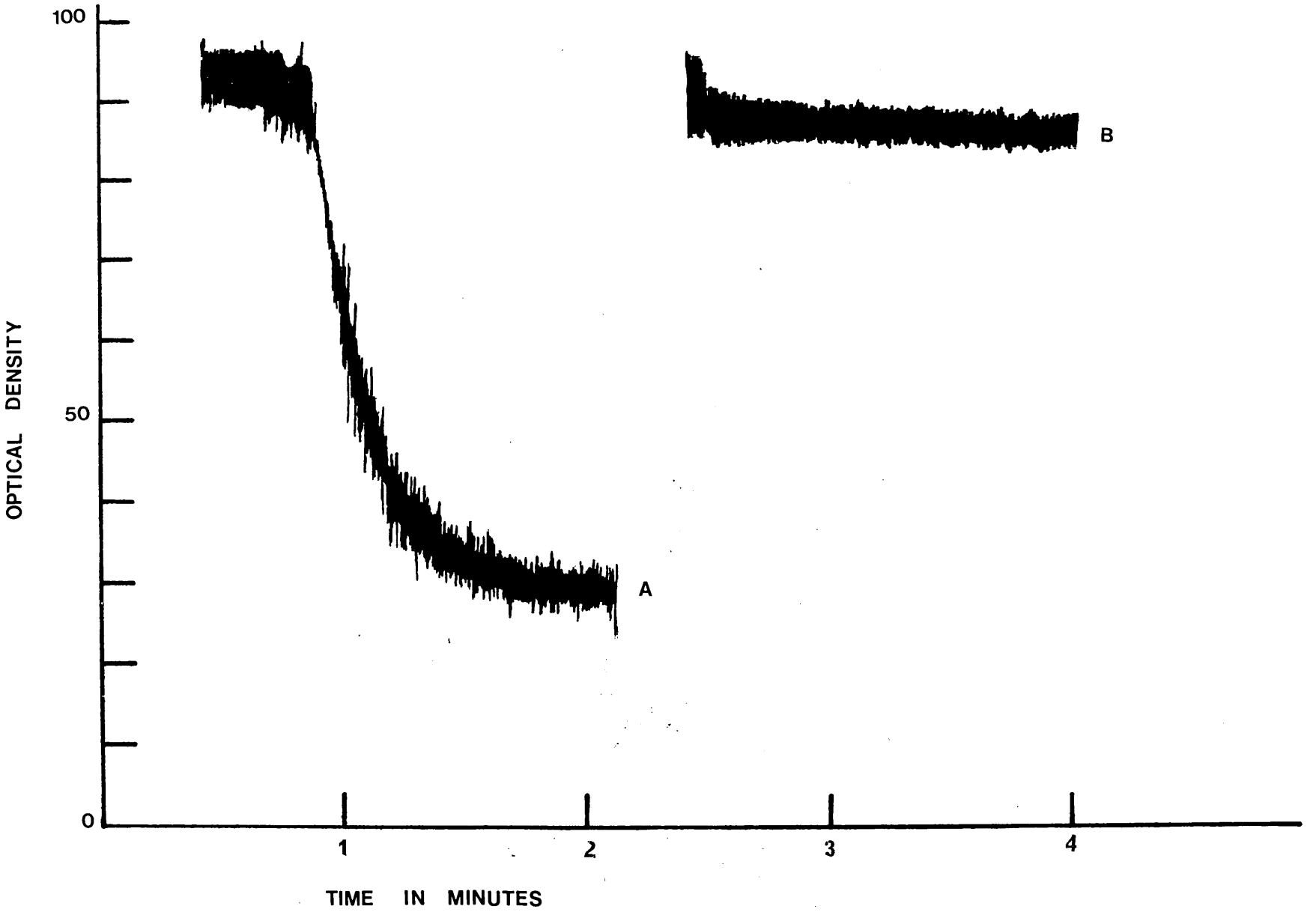
Platelet rich plasma was obtained by the centrifugation of blood at 450 x g for 10 mins. in an MSE Minor centrifuge. The anticoagulant used was 3.13% (w/v) trisodium citrate in the ratio of one volume to nine volumes of blood. After removing the prp, the remainder of the blood was centrifuged at 2,400 x g for 10 mins. to obtain platelet-poor plasma (ppp). The platelet count of the samples was standardised using the optical density method described by Born (197). An EEL aggregation meter, model 169 (Evans Electroselium Ltd., Halstead, Essex) was used at a temperature of 37^{o}C and at a speed of 80 on the arbitrary indicator. The aggregation results were recorded on a Leeds and Northrup flat bed recorder set on 5Mv and a speed of 75cm/hr. The measuring scale on the aggregation meter was marked in arbitrary units of 0-60 which was expanded on the flat bed recorder to 0-100.

Fig. 12 demonstrating the effect of aspirin on collagen aggregation.

"A" shows the normal response with collagen.

"B" shows the response after the patient was given two 300 mg dose of aspirin 24h prior to the blood test.

The concentration of the collagen added was standardised as described by O'Brien (199).


100
50
0
OPTICAL DENSITY
A
B
1
2
3
4
TIME IN MINUTES

The ppp was placed in the aggregating tubes supplied by EEL, positioned in the well of the meter and the scale adjusted to zero using the 'set meter' control. The ppp was removed and replaced by the prp specimen. This was adjusted by the addition of ppp until a reading of between 38 and 42 was obtained on the measuring scale. One ml amounts of the adjusted prp specimens were placed in the aggregation tubes and left at room temperature. The prp specimens were warmed to 37°C for 5 mins. prior to testing and then placed in the well of the aggregation meter and the stirrer positioned in the tube. After 2 mins., 0.1 ml of the "critical dose" of ADP (pre-warmed to 37°C) was added and the aggregation pattern observed on the recorder.

2.2.1 Correlation of platelet numbers. The total platelet count using the Coulter Counter (Coulter Electronics, Luton, Beds.) and the optical density (OD) method of Born (197) were compared. The scale on the aggregating meter was set to zero using ppp, this was removed and replaced with varying concentrations of prp. The readings on the meter scale were noted and recorded. Platelet counts were also performed on the same preparations of prp using the Thrombocounter-C available from Coulter Electronics. The OD readings were compared with the total platelet counts.

2.2.2. Estimation of the 'critical dose" of ADP. A stock solution of ADP (Sigma, Poole, Dorset) was prepared by dissolving 100mg in 200ml 0.06 M phosphate buffered saline (pH 7.4). Dilutions of this stock solution were prepared in this diluent and are listed in Table 2. To estimate the "critical dose" of ADP, 0.1 ml aliquots of the diluted stock were added to 1 ml volumes of prp preparations from normal healthy donors using the method already described. The "critical dose" was the highest concentration of ADP that showed a normal disaggregation pattern.

2.2.3 Collagen induced aggregation. The collagen was prepared

Table 2 showing the dilutions of the stock ADP solution in 0.06 M phosphate buffered saline (pH 7.4).

Dilution of Stock	Neat	1/20	1/30	1/40	1/50	1/60	1/70	1/100
Concentration of ADP in µg/0.1ml	50	2.50	1.67	1.25	1.00	0.83	0.71	0.50

from homogenised, filtered human tendon and standardised as described by O'Brien (199).

2.2.4 Population survey using platelet aggregation patterns. The technique has been used in a preliminary investigation of the normal population. In a Carmarthen community survey carried out by the Medical Research Council Epidemiological Unit (Cardiff), 65 apparently normal healthy men were examined and platelet samples were also obtained from 23 confirmed myocardial infarction patients. In addition, 23 hospital based control patients were studied. Both groups were part of a larger study (200) and diagnosis had been established.

Subjects referred to as myocardial infarction patients (MI patients) were males up to the age of 65 who had suffered what is variably known as "heart attack", coronary thrombosis or myocardial infarction. (Its significance in relation to coronary heart disease has already been detailed in Section 1.1.2 page 22). The only patients included in this work were investigated and diagnosed by a doctor of consultant status. The diagnosis was based upon typical clinical history, unequivocal electro-cardiographic changes and changes in circulating myocardial enzymes diagnostic of infarction following clinical occurrence. The medical consultants involved maintained strict criteria and any cases where the diagnosis was equivocal were excluded. The normal controls from the community were chosen at random from the electoral list and the hospital control patients included those with a variety of diseases. Exclusion in this latter group was restricted to patients:-

(a) with a history of, or evidence of an immunological disturbance, or a condition known to have an immunological basis or relationship,

(b) with any evidence of bleeding.

(c) post operative cases.

(d) clinical evidence of atherosclerosis.

The MI patients in this survey had suffered from the infarction at least twelve months prior to the test. This excluded any

interference from factors present in the circulation which can cause abnormal results in the acute state of the condition. The significance and importance of this time factor has already been discussed on page 15. The tests were performed "blind" with regard to the diagnoses which were revealed by the MRC after the completion of the survey. Specimens were tested in duplicate and the results read and recorded.

2.2.5 Statistical analyses. The following statistical analyses are described in this thesis.

Arithmetic mean. This is best known as average and is defined by the sum of the separate values in a series, divided by their numbers (201). When values of a variable group of numbers are represented by x_1, x_2, x_3....x_n, the mean or average is defined by $\sum_n^{} x$. The Greek capital, "sigma", denotes "the sum of" and the arithmetic mean is represented by $\bar{x}$. n is the total number involved in the calculation.

Standard deviation. This is the measure of dispersion most commonly used in statistics and is a measure of the spread or scatter of the distribution around the mean. In a normal distribution a departure of one standard deviation (SD or S) on either side of the mean will include approximately 68% of the values of a given distribution; two standard deviations from the mean includes about 95% of all values and three standard deviations will include almost all of the distribution values (202). This is illustrated in Fig. 13. When establishing a normal range the values between ± 2 SD are accepted as normal (203). The formula for calculating the standard deviation is

$$SD = \sqrt{\frac{\sum (x-\bar{x})^2}{n}}$$

in which $\bar{x}$ = mean value
$x - \bar{x}$ = deviation from the mean
n = number of specimens tested

The deviations are squared because the problems arising from negative signs are dispensed with. There is one refinement of the standard deviation. If the observations are only a

Fig. 13 illustrating the properties of a normal curve.

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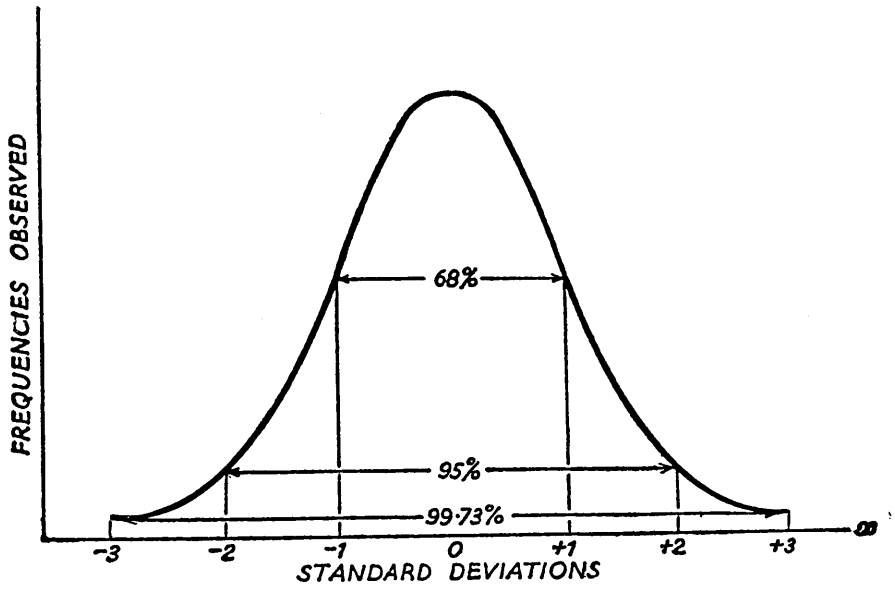
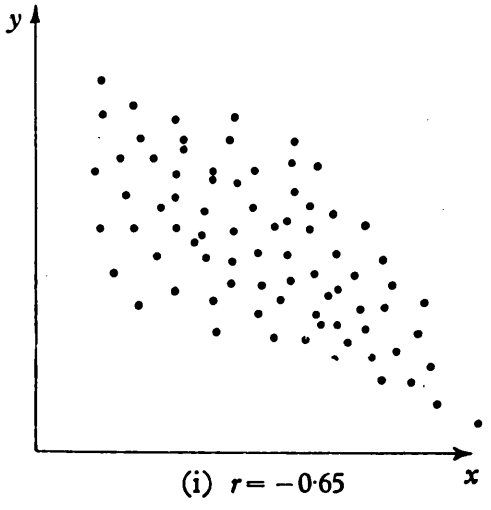
Reichmann, W. J., p.215 (202).

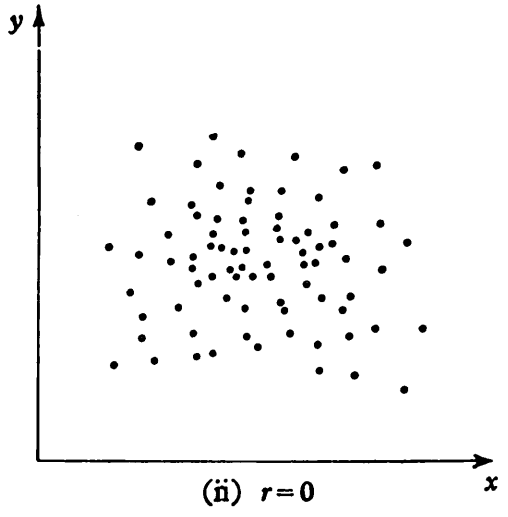
Illustrated opposite are four scatter diagrams giving different values of r. Each dot represents one pair of measurements and the changeover from negative correlation through zero to positive correlation is shown.

Reproduced with permission of the publishers.
Moore, P. G. (1969) in Principles of Statistical Techniques, p.262. Cambridge University Press.

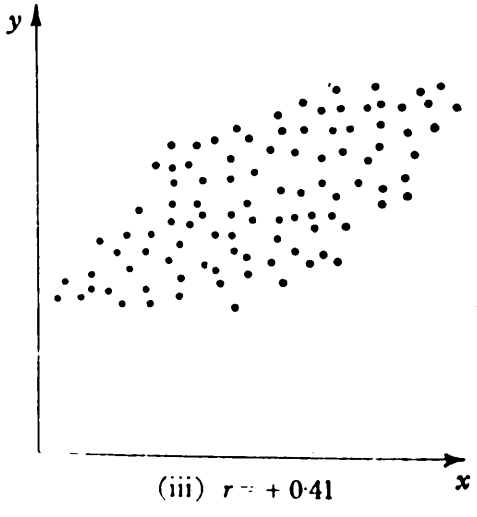




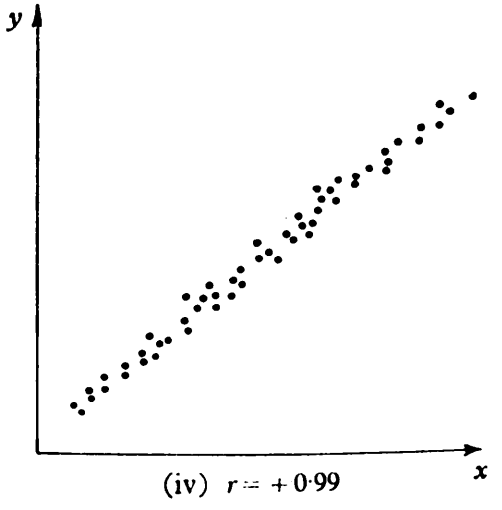
(i) $r = -0{\cdot}65$



(ii) $r = 0$



(iii) $r = +0{\cdot}41$



(iv) $r = +0{\cdot}99$

sample (or sub-set) of a larger set of observations then the devisor is n - 1 and not n. The change is usually a marginal one numerically, but may be important if the sample is small.

Correlation. Correlation techniques in statistics are methods of examining the relationship of one measure to the other. There are varying degrees of correlation which range from 1.0, which is perfect, to 0.0 which represents no correlation. Relationship may be negative or positive. The number which indicates this correlation between two variables is termed "correlation coefficient" which is usually represented by the letter "r". Therefore the value of r reflects the degree by which two variables are related (204). Most automatic calculators calculate r using the following formula:-

$$r = \frac{n.\Sigma xy - \Sigma x.\Sigma y}{\sqrt{\left\{n.\Sigma x^2 - (\Sigma x)^2\right\}\left\{n.\Sigma y^2 - (\Sigma y)^2\right\}}} \qquad (204)$$

x represents one set of figures and y the other.

It is advisable to plot the corresponding values of the variables on a chart. The regression line drawn represents the linear equation to which the related behaviour of the two variables most closely approximates. It is possible to calculate the slope and axial interception of this line using a mathematical formula formulated by the French mathematician Legendre (202). The line may be used to predict the approximate value of one variable, given the known value of the other. However, the regression line may be drawn on a chart merely to demonstrate that correlation exists between two variables and that the correlation is linear and therefore the r value had not been calculated from a narrow range of corresponding figures. When the values of the two variables are plotted on the chart, one set of figures is represented on the abscissa and the other set on the corresponding ordinate. These types of charts are referred to as "scatter diagrams" and are illustrated in Fig. 13. When the points are scattered closely about the regression line, so that the distances between the points and the line are short, there is a clearer possibility of the line actually defining a true relationship. If, however, the points are scattered all over the chart, no line can be drawn

to represent any relationship.

Having obtained a value r for two sets of variables, it is necessary to determine the "degree of significance" of the result. This is reported as "probability" (p) which determines whether the results are not due to chance and is determined from the appropriate mathematical tables. The table used was devised in 1908 by "Student" (the pen name of W. S. Gassett) (201) who established the theoretical distribution of t and this discovery made it possible to calculate a table giving the significant levels of observed values of t. The t-Table used in this thesis is illustrated in Table 3. After obtaining the value for r, the degree of significance (or probability p) can be obtained by the use of the t-table using the following formula.

$$t = \frac{r}{\sqrt{1 - r^2}} \cdot \sqrt{n - 2}$$

n represents the number of pairs of observations on which the correlation is based, r is the correlation obtained and n - 2 the degrees of freedom (df).

The p value can therefore be calculated using Table 3 and hence the significance that can be attributed to the results. When the p value is more than 0.05 it means that there is a greater than 5% chance that the event or occurrence under test could happen by chance alone (p of 0.01 equals a 1% chance, 0.001 equals 0.1% chance etc.). A figure of more than 0.05 is considered unacceptable and the results fail to show correlation. p values between 0.05 and 0.01 suggest that the results should be accepted with some confidence and a p value of less than 0.01 can be accepted with greater confidence.

The SD, $\bar{x}$ and the r values described in the statistical sections of this thesis have been calculated using a "Casio Scientific Calculator" (Casio Electronics, 1000 North Circular Road, London) model fx 3500p using the formulae described in this section.

Table 3 demonstrating the "t-table".

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N·	P=·9 (90%)	·8 (80%)	·7 (70%)	·6 (60%)	·5 (50%)	·4 (40%)	·3 (30%)	·2 (20%)	·1 (10%)	·05 (5%)	·02 (2%)	·01 (1%)	·001 (0·1%)
1	·158	·325	·510	·727	1·000	1·376	1·963	3·078	6·314	12·706	31·821	63·657	636·619
2	·142	·289	·445	·617	·816	1·061	1·386	1·886	2·920	4·303	6·965	9·925	31·598
3	·137	·277	·424	·584	·765	·978	1·250	1·638	2·353	3·182	4·541	5·841	12·941
4	·134	·271	·414	·569	·741	·941	1·190	1·533	2·132	2·776	3·747	4·604	8·610
5	·132	·267	·408	·559	·727	·920	1·156	1·476	2·015	2·571	3·365	4·032	6·859
6	·131	·265	·404	·553	·718	·906	1·134	1·440	1·943	2·447	3·143	3·707	5·959
7	·130	·263	·402	·549	·711	·896	1·119	1·415	1·895	2·365	2·998	3·499	5·405
8	·130	·262	·399	·546	·706	·889	1·108	1·397	1·860	2·306	2·896	3·355	5·041
9	·129	·261	·398	·543	·703	·883	1·100	1·383	1·833	2·262	2·821	3·250	4·781
10	·129	·260	·397	·542	·700	·879	1·093	1·372	1·812	2·228	2·764	3·169	4·587
11	·129	·260	·396	·540	·697	·876	1·088	1·363	1·796	2·201	2·718	3·106	4·437
12	·128	·259	·395	·539	·695	·873	1·083	1·356	1·782	2·179	2·681	3·055	4·318
13	·128	·259	·394	·538	·694	·870	1·079	1·350	1·771	2·160	2·650	3·012	4·221
14	·128	·258	·393	·537	·692	·868	1·076	1·345	1·761	2·145	2·624	2·977	4·140
15	·128	·258	·393	·536	·691	·866	1·074	1·341	1·753	2·131	2·602	2·947	4·073
16	·128	·258	·392	·535	·690	·865	1·071	1·337	1·746	2·120	2·583	2·921	4·015
17	·128	·257	·392	·534	·689	·863	1·069	1·333	1·740	2·110	2·567	2·898	3·965
18	·127	·257	·392	·534	·688	·862	1·067	1·330	1·734	2·101	2·552	2·878	3·922
19	·127	·257	·391	·533	·688	·861	1·066	1·328	1·729	2·093	2·539	2·861	3·883
20	·127	·257	·391	·533	·687	·860	1·064	1·325	1·725	2·086	2·528	2·845	3·850
21	·127	·257	·391	·532	·686	·859	1·063	1·323	1·721	2·080	2·518	2·831	3·819
22	·127	·256	·390	·532	·686	·858	1·061	1·321	1·717	2·074	2·508	2·819	3·792
23	·127	·256	·390	·532	·685	·858	1·060	1·319	1·714	2·069	2·500	2·807	3·767
24	·127	·256	·390	·531	·685	·857	1·059	1·318	1·711	2·064	2·492	2·797	3·745
25	·127	·256	·390	·531	·684	·856	1·058	1·316	1·708	2·060	2·485	2·787	3·725
26	·127	·256	·390	·531	·684	·856	1·058	1·315	1·706	2·056	2·479	2·779	3·707
27	·127	·256	·389	·531	·684	·855	1·057	1·314	1·703	2·052	2·473	2·771	3·690
28	·127	·256	·389	·530	·683	·855	1·056	1·313	1·701	2·048	2·467	2·763	3·674
29	·127	·256	·389	·530	·683	·854	1·055	1·311	1·699	2·045	2·462	2·756	3·659
30	·127	·256	·389	·530	·683	·854	1·055	1·310	1·697	2·042	2·457	2·750	3·646
∞	·12566	·25335	·38532	·52440	·67449	·84162	1·03643	1·28155	1·64485	1·95996	2·32634	2·57582	3·291

2.3. Results

2.3.1 Platelet disaggregation ratio. On the addition of ADP, platelet aggregation occurred and this was measured by the reduction in optical density (mark "A" in Fig. 14) which rapidly returned to "B". A ratio of A/B was used to quantify the "disaggregation ratio". Preliminary work using more than 50 normal subjects and confirmed MI patients showed that a normal disaggregation response appears to be a ratio of 1.9 or less. Prolonged discussions between the MRC statisticians took place to consider the most satisfactory means of quantifying the "disaggregation ratio". It was agreed that measuring the disaggregation "B" at an arbitrary distance 5 cm from "A" provided the most satisfactory discrimination between the confirmed MI patients and "normal" controls (See Fig. 14).

2.3.2 ADP titration. Fig. 15 illustrates typical aggregation patterns which occur after the addition of the various concentrations of ADP. In this illustration a concentration of 1.0µg/0.1 ml is the highest dilution of ADP that demonstrated a normal disaggregation ratio (1.9 or less) with a population of platelets from a normal donor. The stock and working dilutions of ADP were divided into small aliquots and stored at $-20^{o}C$. The titration described was periodically checked and performed on every new batch of ADP.

2.3.3 OD and platelet counts. The correlation obtained between the total platelet count using the Coulter Counter and the optical density method described by Born is illustrated in Fig. 16. The statistical analyses showed that p was highly significant (<0.001), r being 0.73. A reading of between 38 and 42 on the aggregation meter scale, referred to in the method, corresponded to an actual platelet count of between 250 and 350 x $10^{9}/1$.

2.3.4 Population survey and platelet aggregation. The disaggregation results are tabulated in Table 4. The results can be

Fig. 14 demonstrating the "disaggregation ratio".

The aggregation is demonstrated by the reduction "A" in the optical density which rapidly disaggregates to "B".

A ratio of A/B was used to quantify the "disaggregation ratio". Where the ratio has been modified to "percentage disaggregation" the formula $\frac{B}{A} \times 100$ was used.

(ADP concentration = 1.0 µg/0.1 ml. Volume added to 1 ml of platelet rich plasma = 0.1 ml).

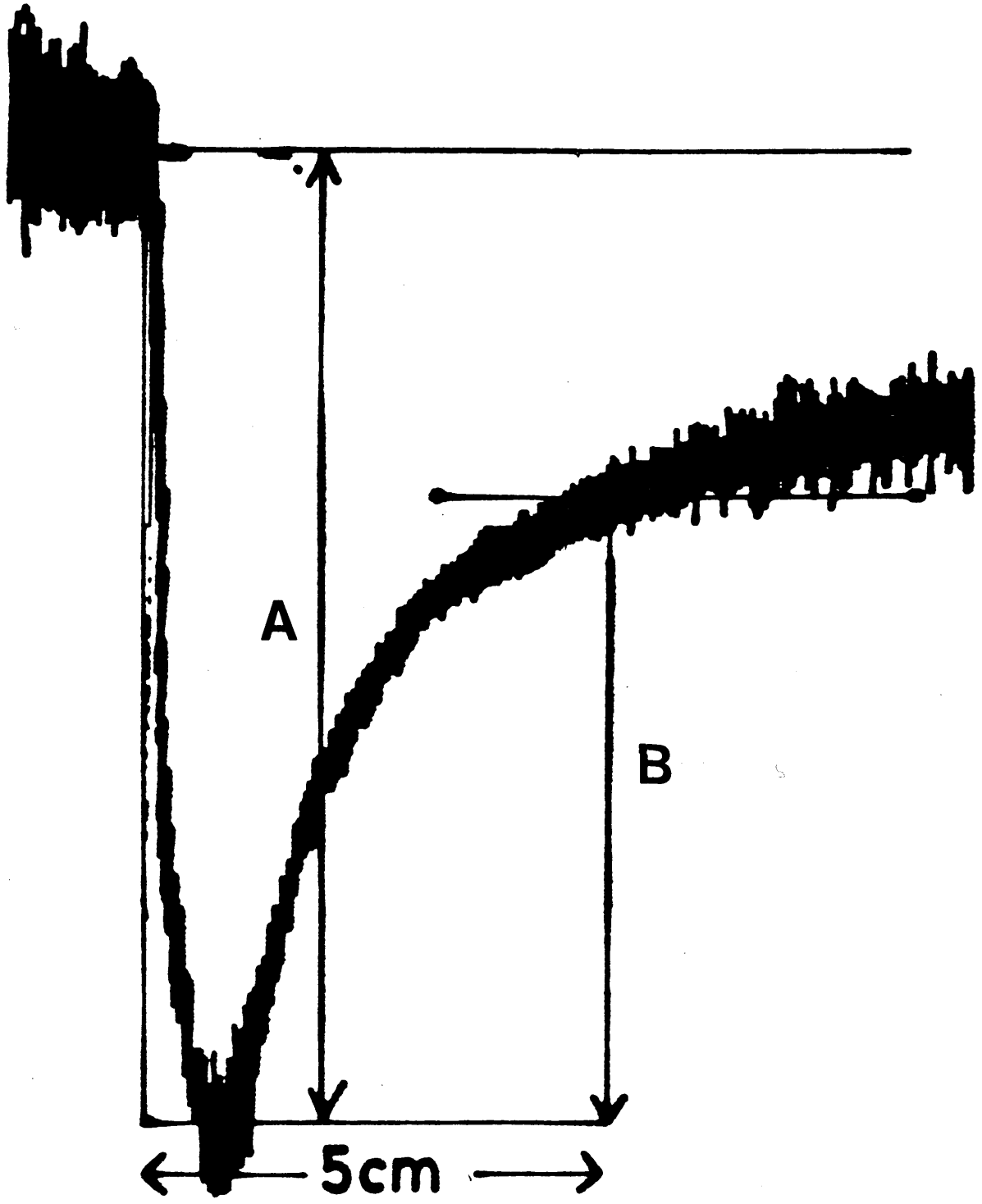

A
B
5cm

Fig. 15 demonstrating typical aggregation patterns obtained after the addition of the various concentrations of ADP.

Response curve "D" shows a ratio of 2.1 and curve "F" a ratio of 1.8. Therefore, the highest concentration of ADP that shows a normal response of 1.9 is curve E (ADP concentration of 1.0 µg/0.1 ml.)

The concentrations of ADP were as follows:-

A = 50 µg/0.1 ml
B = 2.50 µg/0.1 ml
C = 1.67 µg/0.1 ml
D = 1.25 µg/0.1 ml
E = 1.00 µg/0.1 ml
F = 0.83 µg/0.1 ml

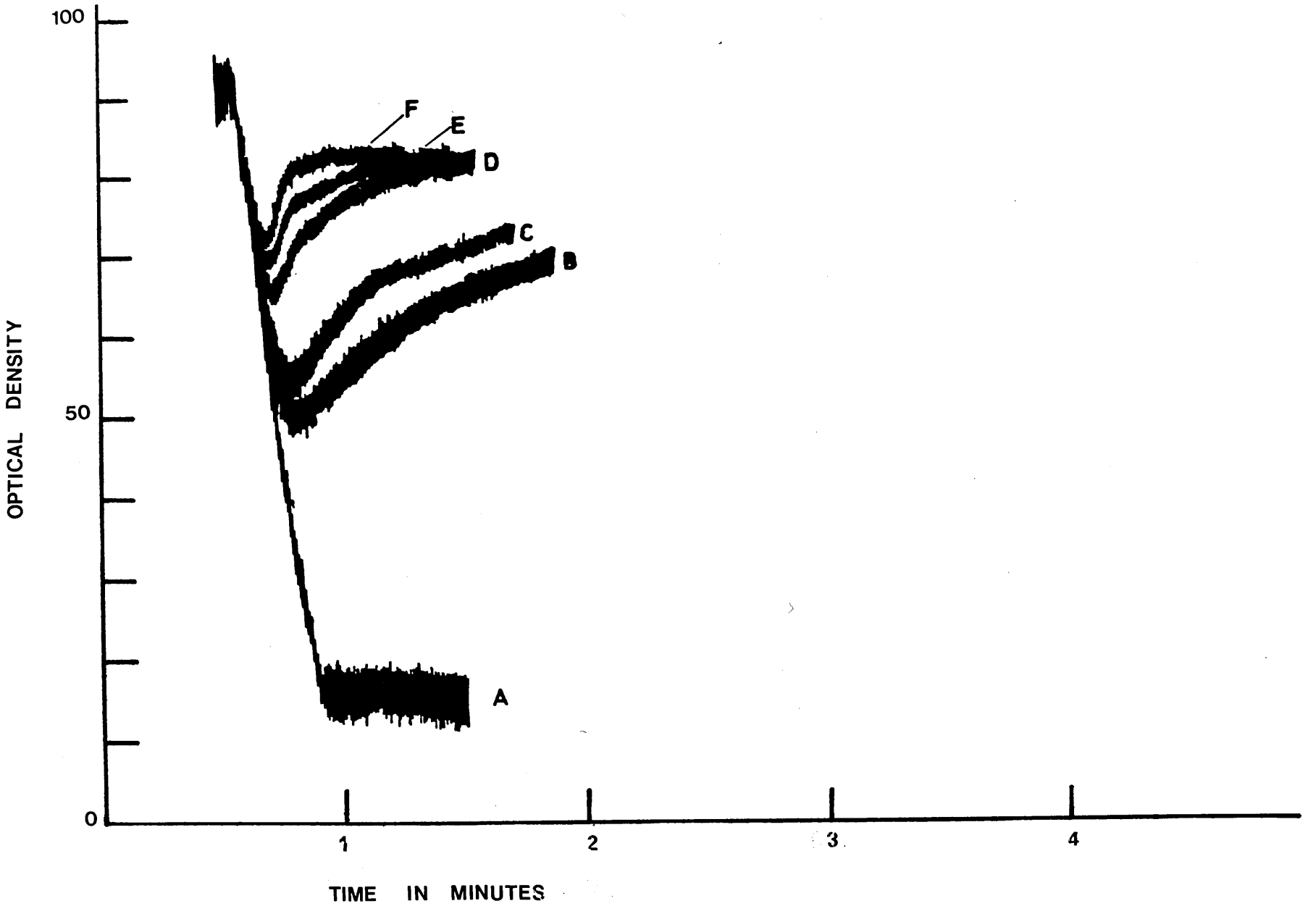

100
50
0
OPTICAL DENSITY
F
E
D
C
B
A
1
2
3
4
TIME IN MINUTES

Fig. 16 demonstrating the correlation obtained between the platelet count performed on the Coulter Counter and the optical density method described by Born (197). n = 64. r = 0.73p = < 0.001.

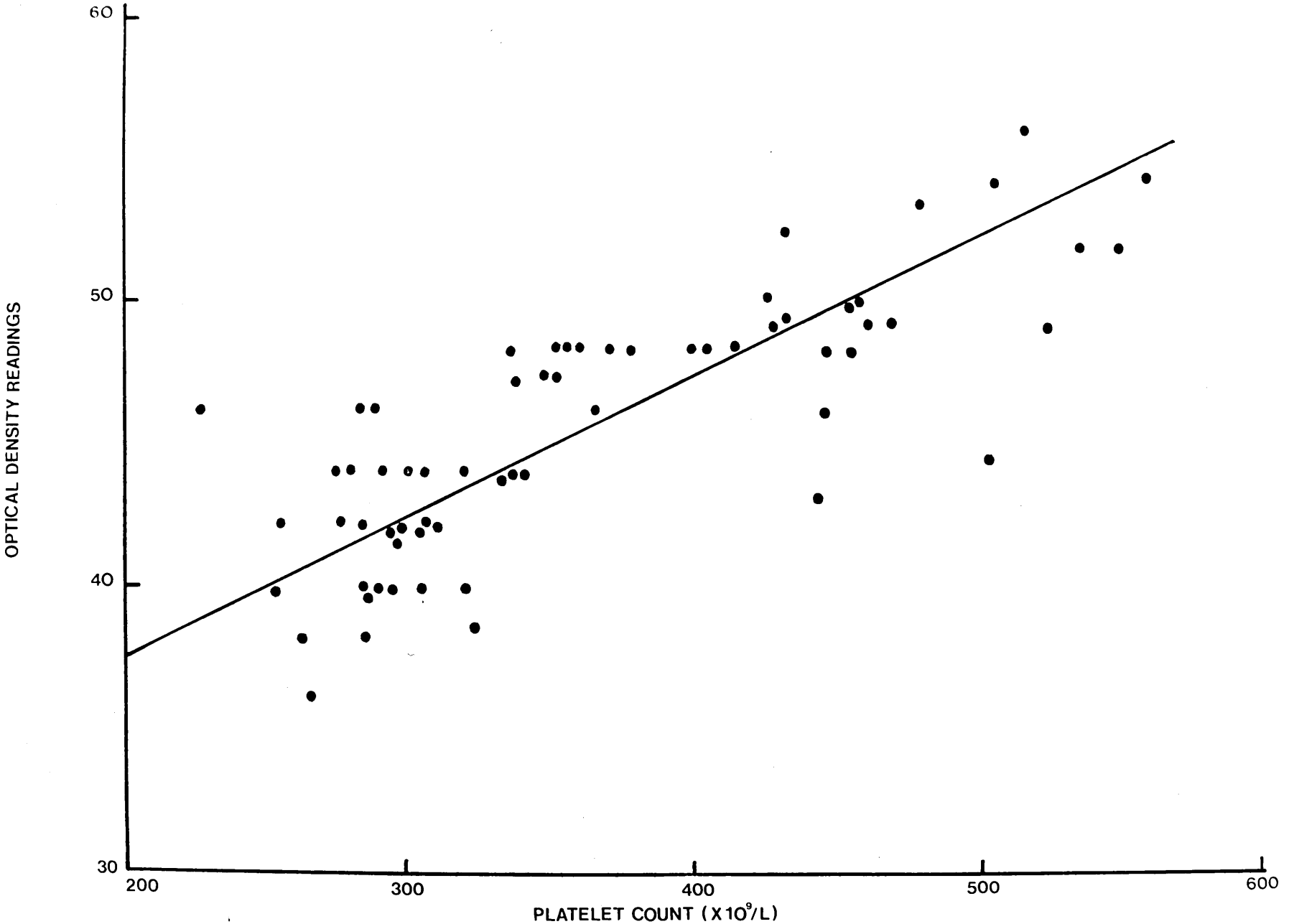

60
50
40
30
OPTICAL DENSITY READINGS
200
300
400
500
600
PLATELET COUNT (X10⁹/L)

Table 4 showing the distribution of "disaggregation ratios" in the community survey.

Ratio	Numbers of Patients		
	Community Controls	Hospital Controls	MI Patients
1.4	13	7	0
1.4	5	1	0
1.5	4	1	1
1.6	6	5	1
1.7	9	2	1
1.8	8	3	0
1.9	8	1	0
2.0	3	1	0
2.1	1	1	0
2.2	1	0	0
2.3	1	0	0
2.4	1	0	1
2.5	0	0	1
2.6	1	1	0
2.7	1	0	0
3.0 - 10.0	2	0	6
11.0 - 20.0	1	0	0
21.0 - 30.0	0	0	2
31.0 - 100.0	0	0	1
100.0 +	0	0	9
Totals	65	23	23

Only subjects with a normal response to collagen are included

summarised as follows:-

1. Of 65 males in the community survey, 12 exhibited an abnormal ratio of greater than 1.9 i.e. 18.5% showed abnormal disaggregation.

2. Of the hospital based controls, 3 out of the 23 patients tested showed abnormal ratios i.e. 13% showed abnormal disaggregation.

3. Of the MI patients who had suffered the infarct at least 12 months previously, 20 out of 23 subjects exhibited an abnormal ratio i.e. 87% showed abnormal disaggregation.

2.4 Discussion

It was found that the sensitivity of the method developed was maximal if the ADP was used at the concentration termed the "critical dose". This was the highest concentration that allowed normal disaggregation in a population of platelets from normal donors. It was obtained by progressively increasing the concentration of ADP until normal disaggregation no longer occurred. A ratio was devised to reflect the degree of disaggregation and subsequent experiments confirmed that a ratio of 1.9 or less discriminated well between known MI patients and normal controls. When this "critical dose" of ADP was added to platelet rich plasma from certain type of donors, it was found that irreversible aggregation occurred. It was considered that this phenomenon was due to the presence of a higher percentage than normal of "active" platelets in the circulation of these subjects.

The disaggregation ratio patterns obtained in the experiments performed to date can be classified into three main groups. These are illustrated in Fig. 17. Aggregation (a) is a typical pattern found in normal control subjects. Pattern (b) illustrates the disaggregation result found in the majority of "long term" confirmed MI patients. It will be

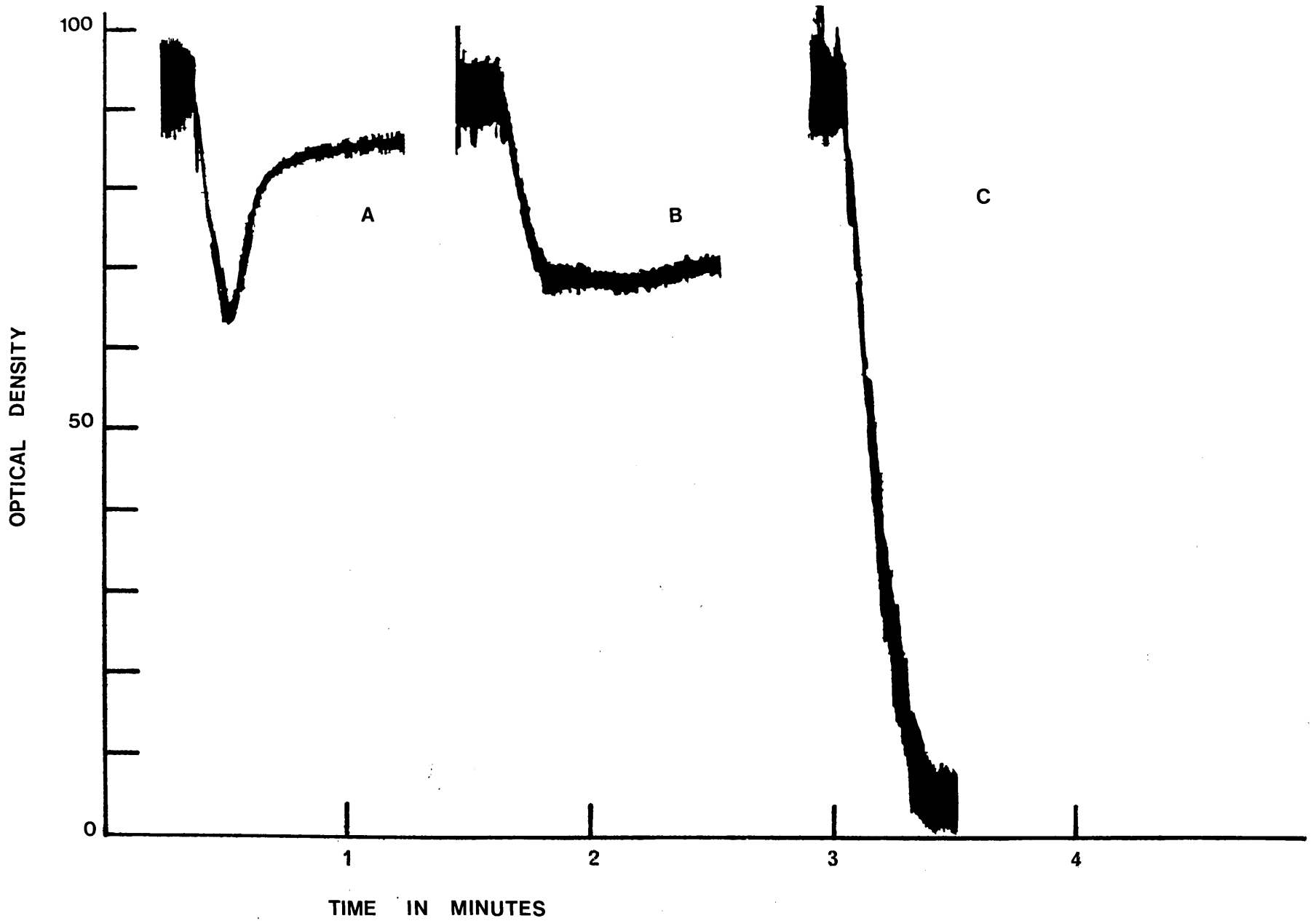
Fig. 17 showing typical disaggregation patterns.

Pattern "A" is from a normal control subject. The disaggregation ratio was 1.4 and the percentage disaggregation 71.4%.

Pattern "B" is a typical disaggregation pattern found in the community based "long term" confirmed MI patient. The disaggregation ratio was 58 and the percentage disaggregation 0%.

Pattern "C" is a typical pattern found in the hospital based acute MI patient. The disaggregation ratio was 210, and the percentage disaggregation 0%.

(ADP concentration = 1.0 µg/0.1 ml,
Volume added to 1 ml of platelet rich plasma
= 0.1 ml).


100
50
0
OPTICAL DENSITY
A
B
C
1
2
3
4
TIME IN MINUTES

noted that the actual aggregation, reflected in the fall in the OD, is the same as that exhibited by a normal population of platelets, however the MI patients differ in the degree of disaggregation. (c) illustrates the typical pattern found in MI patients in the hospital wards in the acute phase of the disease.

It has been suggested that the "disaggregation ratio" should be changed to "percentage disaggregation" in order to facilitate easier statistical analyses. However, the author believes that the ratio reflects the degree of aggregation which appears to relate to the activity of the platelet population. This is illustrated in Fig. 17. If percentage disaggregation was used, both patients (b) and (c) would be reported as 0% disaggregation. However, patient (c) was an acute case which is reflected in the increased fall exhibited. This gross abnormality would have been obvious if the results were reported as "aggregation ratios". Therefore, where appropriate, some of the statistical analyses performed in this study have been calculated using the "disaggregation ratio" and "percentage disaggregation".

The test was used to investigate community and hospital based normal subjects together with "long term" confirmed MI patients. All the tests were performed "blind" and the relevant data supplied by the Medical Research Council after the completion of the surveys. The surveys described revealed the following information which is considered for more detailed discussion in the "General Discussion" section which comprises the last chapter of this thesis.

1. The test developed detected 87% of the confirmed MI patients tested in this "blind" survey performed in conjunction with the MRC. This has been repeated in subsequent surveys performed in the author's laboratory.

2. The community controls were normal healthy males,

examined and interviewed by the MRC staff. Of the community based normal subjects 18.7% showed an abnormal disaggregation ratio. Morris (205) estimates that approximately 25% of men are "affected by major or minor clinical or sub-clinical, chronic or passing coronary heart disease".

CHAPTER 3

ADP DISAGGREGATION AND "ACTIVE" PLATELETS

3.1 Introduction

This chapter investigates the theory that the abnormal disaggregation results obtained after the addition of a "critical dose" of ADP were the result of platelets being stimulated to release endogenous ADP. The theory, which has been detailed on page 27, relates these abnormal results to the presence of an increased number of "active" platelets. Four different methods were used. Two of these four methods were designed to investigate the instrumentation and aggregation method used and the remaining two were to investigate the theory.

The results obtained using the EEL aggregation method were compared with an aggregation meter loaned by ADG (ADG Instruments Ltd., Macon House, 131 High Street, Hitchin, Herts.). This aggregation system was also based on the original technique described by Born (197) and the principle of the method has been outlined on page 5. The ADG system differed in many aspects from the EEL aggregation meter and was therefore particularly useful for inclusion in this experiment. The differences were dependent upon three main factors:-

(a) The speed and method of stirring. It is necessary to stir the platelet-rich plasma with the selected aggregating agent for subsequent aggregation to occur (42, 206). This stirring process potentiates platelet collision which results in aggregate formation. Mustard and Peckard (162) describe how the rate at which aggregation proceeds can be directly related to the rate of stirring and Sexina (207) states that a speed greater than 1,200 revolutions-per-minute (rpm) leads to increased disaggregation. Coller and Gralink (208) and others (209) have studied the mixing of platelet-rich

plasma specimens and have shown that the relationship of mixing ability to stir-bar geometry and mixing speed is shown in the equation $Q \propto NL^2W$, where Q is the mixing ability, N is the rate of stirring, L is the length and W the width of the steel bar. The rate and method that the platelets are stirred is therefore important and related to the aggregation and also disaggregation patterns obtained. The ADG system incorporated a small magnetic rod in the sample which rotated freely by means of a magnetic stirrer as opposed to a rotating fixed vertical rod in the EEL aggregation meter. The EEL meter aggregated platelets using a laminar flow which was proportional to the speed of the rod and the former induced aggregation using a turbulent current.

(b) The shape and size of the cuvettes used by the two systems were different and this is considered to be one important factor that influences the rate of platelet aggregation (207).

(c) The total volume of prp required for the ADG system was 0.2 ml in comparison with 1 ml on the EEL aggregation method.

The effect of a different aggregating agent was compared with the results obtained with the "critical dose" of ADP. "Active" platelets show a rapid response when stimulated by aggregating agents. Therefore, from this hypothesis, abnormal results should be observed when the same population of platelets are stimulated by other aggregating agents. One suitable agent was adrenaline which has been used by O'Brien (199). He found that in MI patients the characteristic two-phase aggregation response showed an abnormal "lag phase".

The theory that the abnormal disaggregation patterns found in certain subjects was due to an increased number of

"active" platelets present being stimulated to release endogenous ADP was also investigated. This experiment was performed by administrating ASA to the patients exhibiting abnormal disaggregation patterns.

As described on page 28, ASA has the effect of blocking the formation of thromboxane A_2 and therefore no dense granules are released from the platelets. Therefore, the administration of ASA should prevent the release of endogenous ADP and the disaggregation patterns should return to normal.

The theory described above implies that the abnormal disaggregation patterns obtained are the result of platelets releasing endogenous ADP. As detailed on page 27, it will also follow that this reflected the presence of an abnormal number of "active" platelets present in the circulation of such patients. However, all the tests as yet described are "indirect" tests where the end results reflect the effect of ADP released by the platelets. Electron microscopy studies were performed to develop these observations by the "direct" examination of the platelet morphology.

The method described in this study was developed from the original work of Shatz and Riddle (126). These workers used electron microscopy to show that platelets change shape immediately after blood is removed from the circulation and demonstrated that these changes were the morphological expression of platelet activation. It was considered that the activity of the platelet population was reflected in the degree of fusion of the pseudopodia after activation by glass. The "active" platelets showed this response whereas the less active cells remained basically round and compact. This is illustrated in Fig. 18.

3.2 Materials and methods

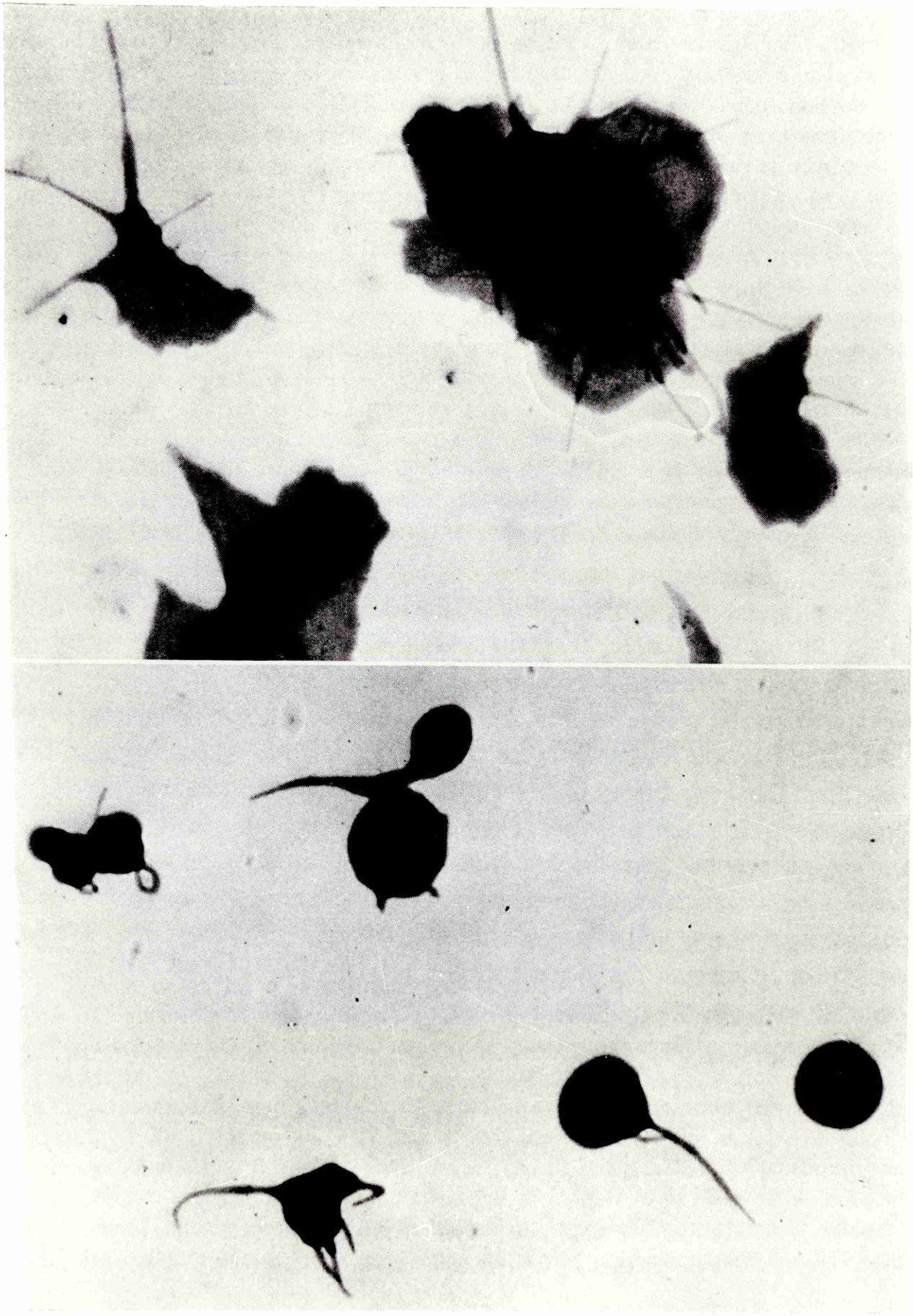
3.2.1 Comparison of aggregation meters. The platelet-rich

Fig. 18 showing transmission electron microscope studies of platelets.

(Top) Response of "active" platelets after 8 mins. activation with glass. The fusion of the pseudopodia reflects the degree of activity.

(Bottom) Platelet preparation showing a normal response after activation with glass.

Reproduced with permission of the publishers.
From Shatz and Riddle (1970), p.146, (126).



plasma specimens were prepared as previously described on page 28 and the same procedure of performing the aggregation tests was followed. The platelet-rich plasma samples prepared were divided into 1 ml aliquots for the EEL system and 0.2 ml amounts for the ADG meter. The two methods were tested in parallel, the amounts of ADP were added and the resulting patterns were read simultaneously on the two-pen recorder used. The disaggregation ratios were calculated and recorded.

3.2.2 Comparison of aggregating agents. Blood was obtained from a number of subjects including some community based confirmed MI patients. The ADP disaggregation tests were performed by the EEL aggregation system using the method outlined on page 28. The adrenaline aggregation test followed the method of O'Brien (199). Preliminary work showed that the hospital based MI patients demonstrated the same type of result as described by O'Brien (199). The community based MI patients, although exhibiting an abnormal ADP disaggregation pattern, did not differ from the normal controls in their response to the adrenaline.

Further experimental work involved a search for a more suitable aggregating agent and it was found that satisfactory results were obtained using the adrenaline issued by the hospital Pharmacy Department. This is commercially available and issued in sealed vials in a liquid form containing 1.8 mg/ml of adrenaline acid tartarate. This is equivalent to approximately 1 mg of adrenaline per ml. The ADP and adrenaline were tested in parallel on two aggregating meters. The volume of reagents used for the adrenaline test was as described on page 28 for the ADP aggregation. The results were read simultaneously on a two-pen recorder.

3.2.3 The effect of ASA on the ADP disaggregation patterns. Blood was obtained from a number of subjects and the ADP disaggregation test was performed as previously outlined. The subjects showing abnormal disaggregation were investigated

three days later as also was an equal number of subjects showing a normal response. This latter group was considered to be a control group.
Two 300 mg doses of ASA were given to the group showing the abnormal response. The ADP disaggregation and collagen tests were repeated on blood samples obtained 24h later.

3.2.4 Electron microscopy studies. The test described on page 40 which was developed by Shatz and Riddle involved adding blood, anticoagulated with 3.13% (w/v) trisodium citrate, to a glass slide at a temperature of 37°C. This was left for exactly 8 min for the glass to activate the platelets before the preparations were washed and fixed. The next stages involved processing the platelets for examination by the transmission electron microscope.

This study describes the development of a method suitable for use using a scanning electron microscope (SEM). Nine volumes of blood was anticoagulated with one volume of 3.13% (w/v) trisodium citrate and treated in two ways:-

(1) During development work, 0.3 ml volumes of this whole blood were added to a series of glass coverslips (prewarmed) and left at 37°C for periods of time which varied from 3-30 mins.

(2) The whole blood specimen was immediately centrifuged at 450 x g for 10 mins. and 0.3 ml volumes of the platelet-rich plasma added to a series of glass coverslips and left at 37°C for the appropriate time. It was found that a platelet count of approximately $200 \times 10^9/l$ gave satisfactory preparations and therefore the prp specimens were diluted with ppp in order to achieve the appropriate count.

The platelets were activated by the glass coverslip and the degree of activation was demonstrated under the SEM. The

method of preparing the platelets for the SEM was based on the method used by Wollweber and his co-workers (210). After preliminary work, the technique was modified (see page 45) and the following method was developed and used to examine platelets under the SEM.

After incubation at 37°C, the excess blood or platelet-rich plasma was carefully removed from the coverslip. Following this, the coverslips were:-

1. Transferred directly to 4% (v/v) paraformaldehyde for 15 mins. at room temperature.
2. Washed in 0.06 M phosphate buffer saline (pH7.2) (PBS).
3. Fixed in 2% (v/v) glutaraldehyde in PBS for 1 h at 4°C.
4. Washed in PBS.
5. Post fixed in 1% (w/v) OSO_4 in veronal acetate buffer (pH 7.2) for 1 h at room temperature.
6. Extensively rinsed in the veronal acetate buffer.
7. Incubated with 1% (w/v) tannic acid in 0.05 M cocodylate buffer (pH 7,2) for 1 h at room temperature.
8. Washed extensively in 0.9% (w/v) sodium chloride.
9. Incubated in 0.5% (w/v) uranyl acetate in double distilled water for 1 h at room temperature.
10. Rinsed in 0.9% (w/v) sodium chloride.
11. Dehydrated in a graded series of acetone solutions (30%, 50%, 70%, 80%, 90% and twice in 100%).

After dehydration the samples were "critical point" dried with CO_2. The coverslips were mounted on brass stubs, gold coated and examined under "Cambridge S150 Scanning Electron Microscope".

Blood specimens were obtained from normal controls, MI patients who had suffered infarction at least 12 months previously and also hospital based acute MI patients. The platelets were prepared and processed for the SEM using the method described and the ADP disaggregation test performed as outlined

on page 28. The platelet disaggregation ratios were calculated and compared with the platelet activity observed by direct examination of the SEM preparations.

3.3 Results

3.3.1 Correlation between the EEL and ADG aggregation meters. Examples of the aggregation charts will be seen in Fig. 19. As illustrated, the aggregation results are similar using both systems but it is noted that the relative size of the patterns obtained on the ADG meter were smaller than those obtained on the EEL. The scatter diagram illustrating the correlation between the two systems is illustrated in Fig. 20 and the results obtained, including the statistical analyses, are listed in Table 5.

3.3.2 Correlation between the ADP and adrenaline. Examples of the results obtained are illustrated in Fig. 21. The patterns demonstrate that, when the ADP disaggregation ratios were normal, no response was observed after the platelets were stimulated with the commercially available adrenaline used in this experiment. In addition, there appeared to be a correlation between the response to this adrenaline and the degree of abnormality of the ADP disaggregation ratios. In order to facilitate statistical analysis, a method was introduced to measure the response to adrenaline. This involved measuring the vertical fall in mm on the chart at an arbitrary distance of 5 cm across from the point where the aggregating agent was added. This is illustrated in Fig. 22. All the 50 normal ADP aggregation ratio results demonstrated no response to the adrenaline. It was observed from the patterns obtained that every abnormal ADP disaggregation ratio showed some degree of response to this adrenaline. Statistical analyses have only been performed on the specimens demonstrating an abnormal disaggregation ratio. The scatter diagram showing the ADP disaggregation ratios and the response to adrenaline in mm is illustrated in Fig. 23.

Fig. 19 showing the correlation between the response of platelet-rich plasma specimens to the "critical dose" of ADP tested simultaneously on an EEL and ADG aggregation system.

"X" Aggregation patterns obtained with the ADG system.

"Y" Aggregation patterns obtained with the EEL system.

(ADP concentration = 1.0 µg/0.1 ml,
Volume added to 1 ml of platelet rich plasma
= 0.1 ml).

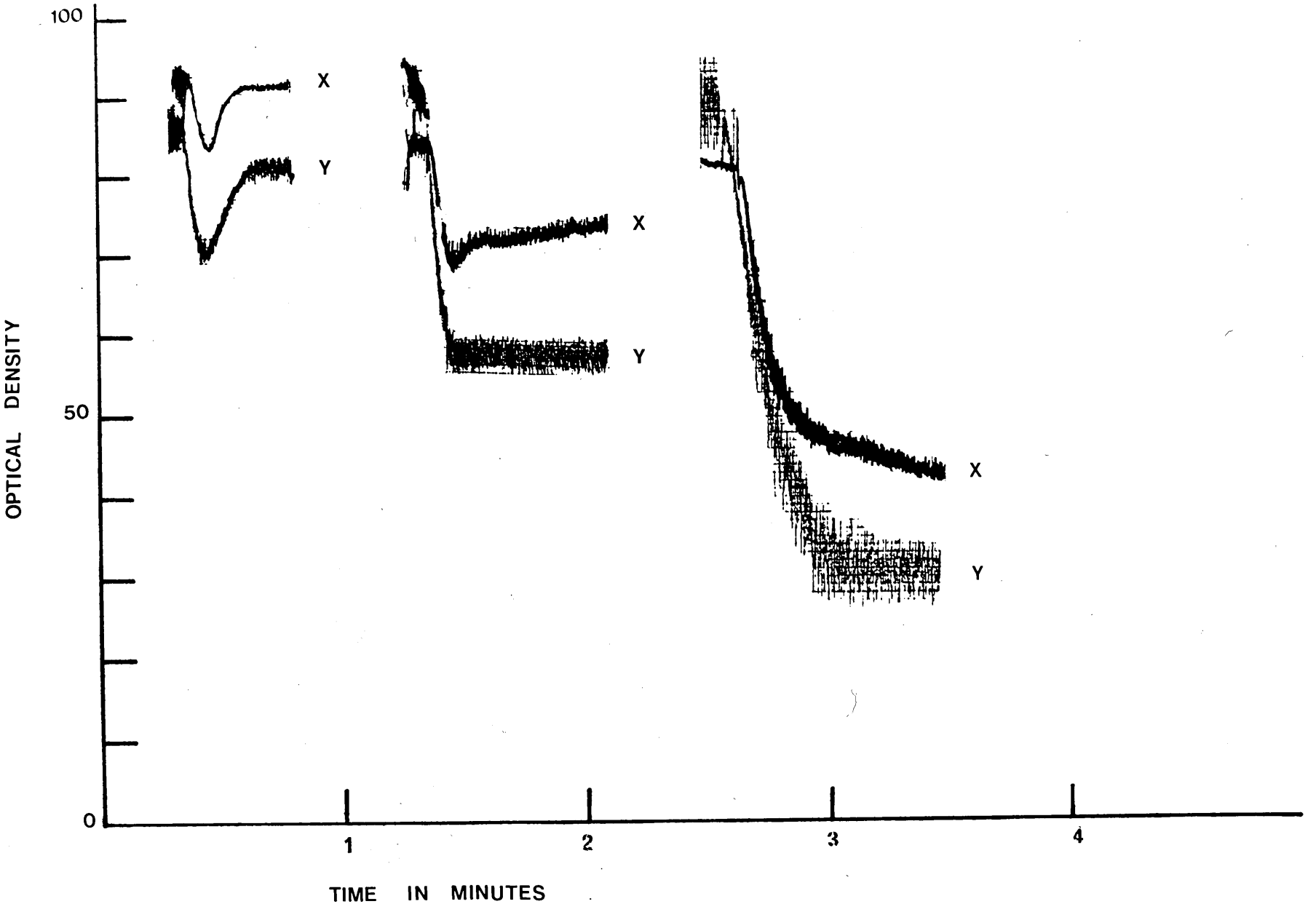

100
50
0
OPTICAL DENSITY
X
Y
X
Y
X
Y
1
2
3
4
TIME IN MINUTES

Fig. 20 Scatter diagram illustrating the correlation between the EEL and ADG aggregating meters.

$n = 24$ $r = 0.97$ $p < 0.001$

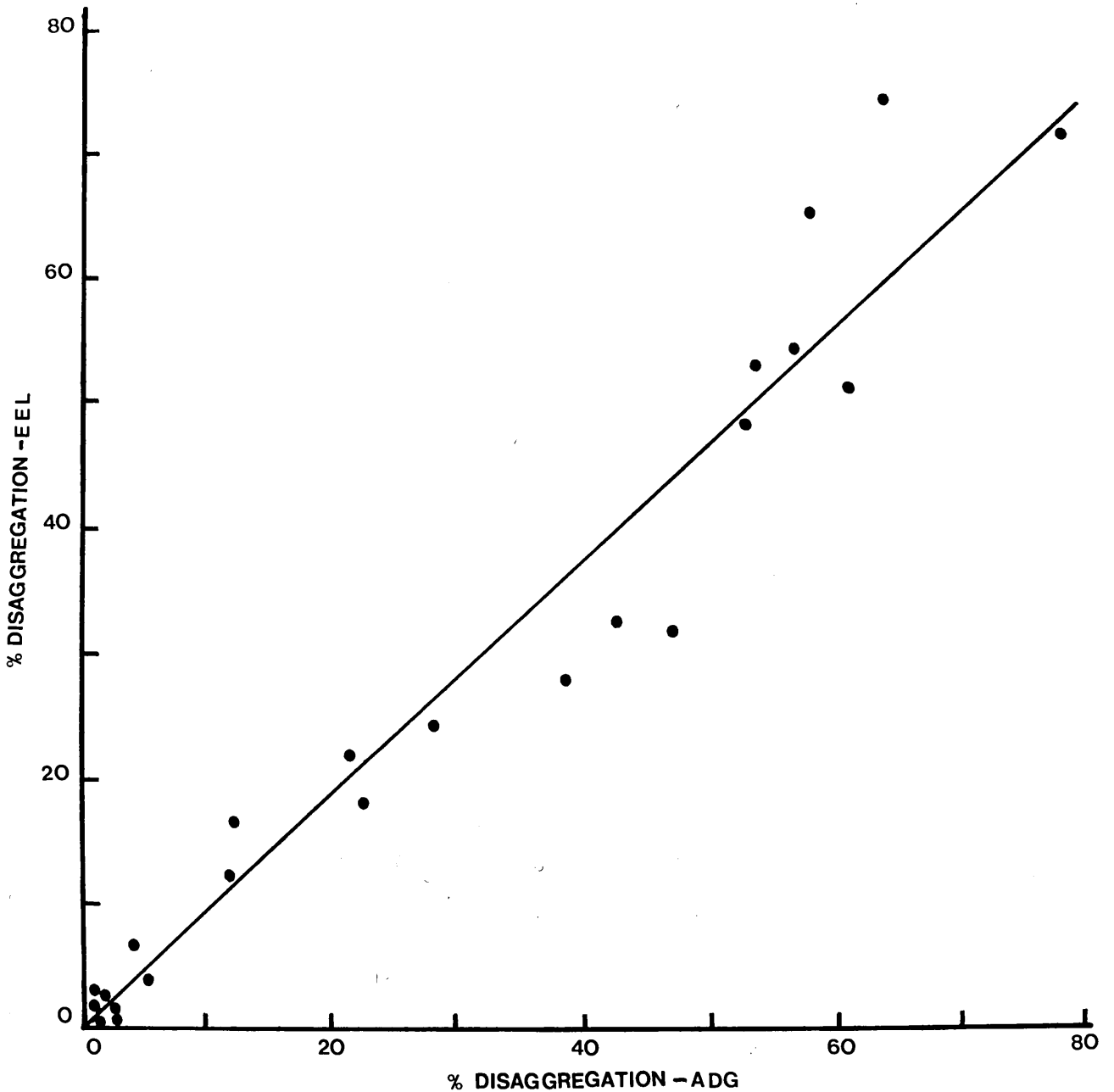

80
60
40
20
0
% DISAGGREGATION - EEL
0
20
40
60
80
% DISAGGREGATION – ADG

Table 5 showing the correlation between the EEL and ADG aggregation meters.

Significant correlation was obtained ($p = < 0.001$) when comparing the "disaggregation ratio" obtained on the two instruments ($r = 0.91$). Similarly when the "percentage disaggregation" results were compared $r = 0.97$.

DISAGGREGATION RESULTS				
	EEL		ADG	
	Ratio	Percentage	Ratio	Percentage
1	1.4	73.7	1.4	78.3
2	80.0	0	3.5	2.8
3	4.0	21.4	4.7	21.2
4	150.0	0	98.0	0
5	3.1	32.6	2.4	42.1
6	80.0	0	47.0	0
7	82.0	0	70.0	0
8	4.1	24.2	3.5	29.0
9	3.5	27.9	2.5	39.0
10	220.0	0	180.0	0
11	125.0	0	140.0	0
12	100.0	0	62.0	0
13	9.0	11.1	8.3	12.0
14	21.0	4.6	20.0	5.0
15	16.4	6.1	24.7	4.1
16	1.9	51.8	1.6	60.1
17	4.7	18.5	4.2	23.8
18	6.0	16.6	7.3	13.6
19	3.3	30.2	2.1	46.7
20	2.1	48.9	1.9	52.0
21	1.9	53.3	1.8	56.6
22	1.5	64.3	1.7	58.0
23	1.9	52.9	1.9	53.3
24	1.3	75.0	1.6	63.8
Mean	34.3	25.5	28.8	27.6
Standard Deviation	53.5	24.8	47.4	25.3

Correlation (r) between the "percentage disaggregation" = 0.97 t = 14.2 $p = < 0.001$

Correlation (r) between the "disaggregation ratio" = 0.91 t = 10.2 $p = < 0.001$

Fig. 21 showing examples of the correlation between the response of platelet-rich plasma specimens the "critical dose" of ADP and adrenaline.

"X" Aggregation with the "critical dose" of ADP.

"Y" Aggregation with adrenaline.

The ADP was used at a concentration of 1.0 µg/0.1 ml and the adrenaline 100 µg/0.1 ml.

The volume added to the platelet rich plasma preparations was 0.1 ml.

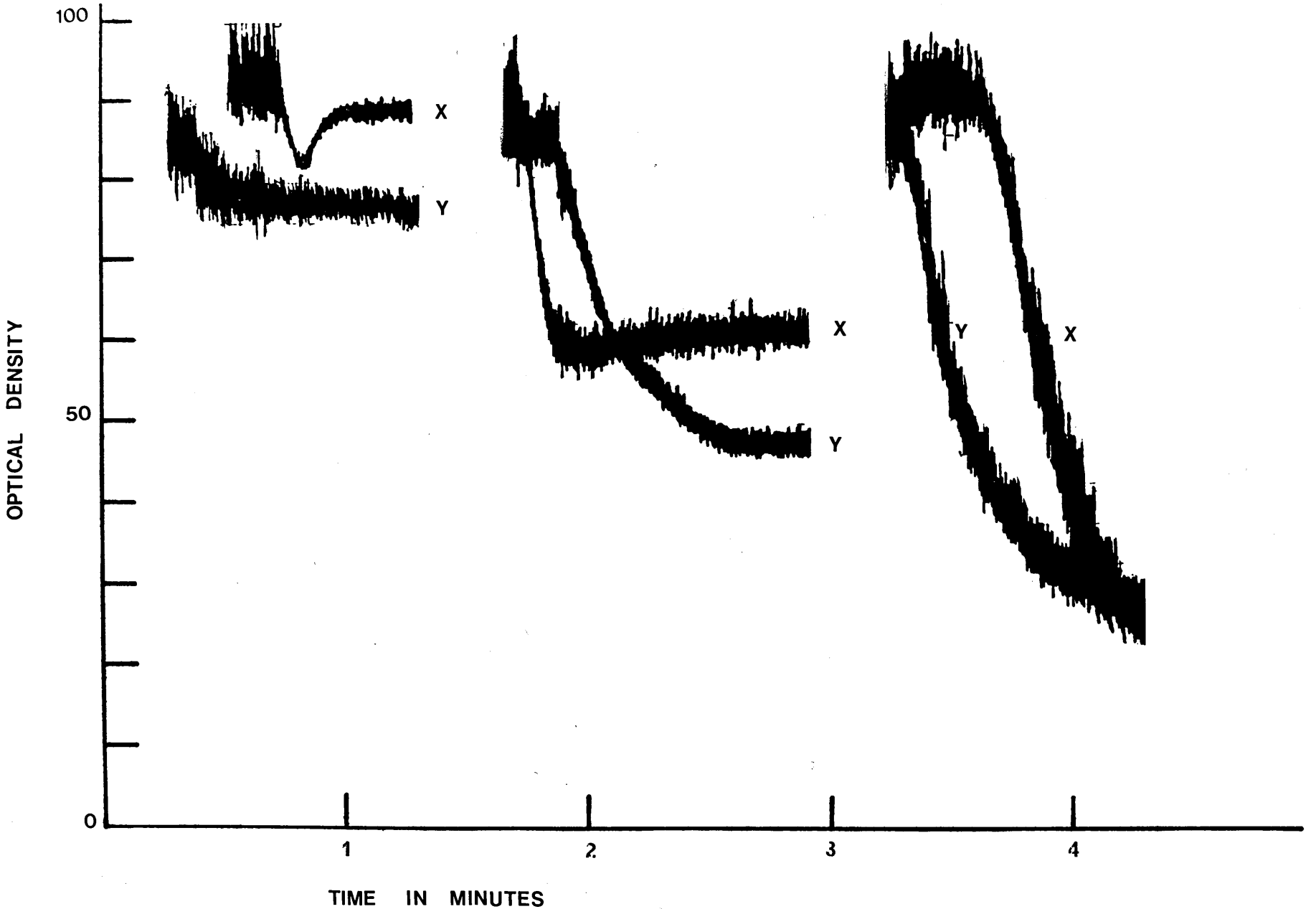

100
50
0
OPTICAL DENSITY
X
Y
X
Y
Y
X
1
2
3
4
TIME IN MINUTES

Fig. 22 showing the method used to represent the aggregation demonstrated by adrenaline. The figure of 5 cm is an arbitrary measurement.

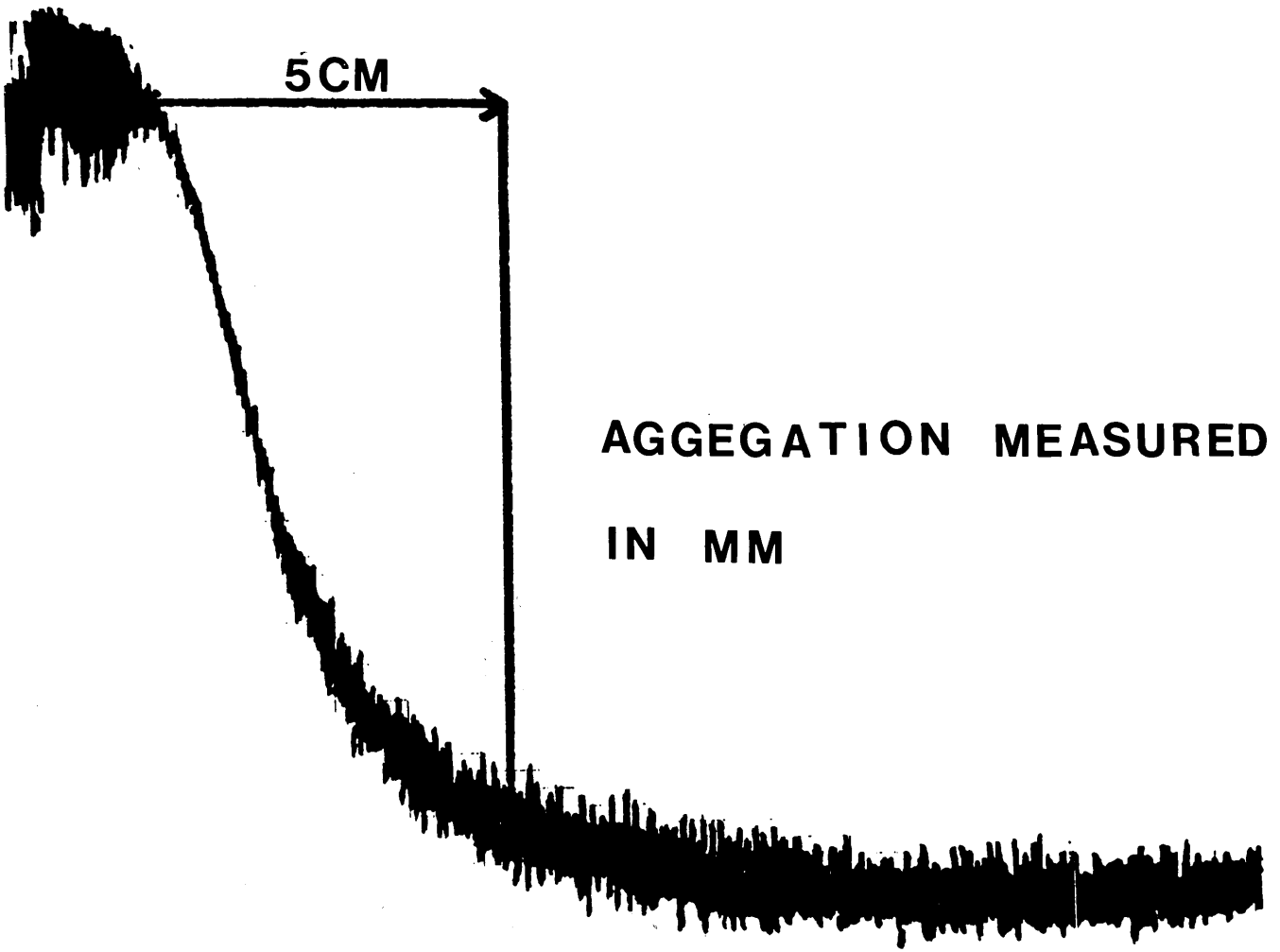
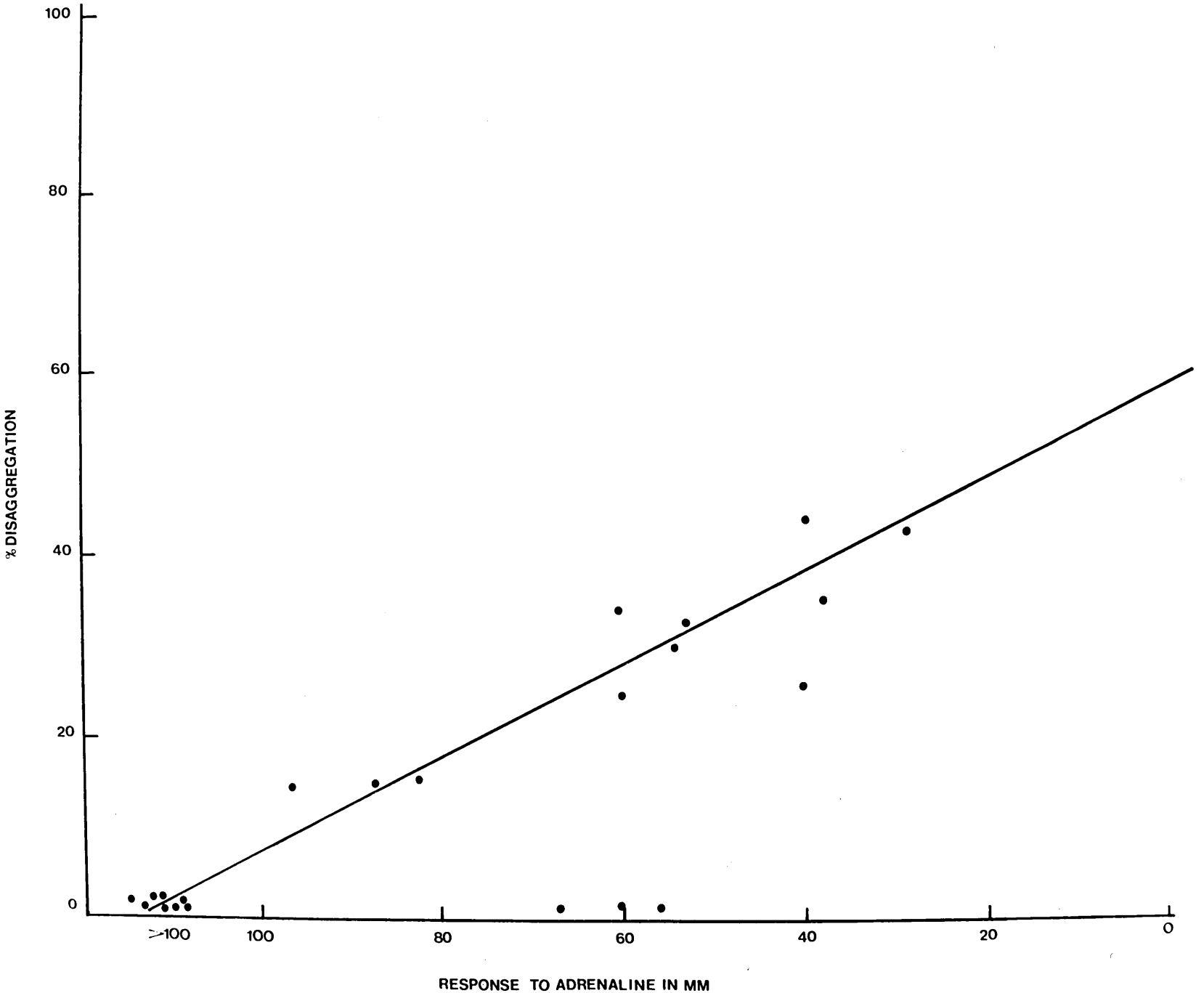

5CM
AGGEGATION MEASURED
IN MM

Fig. 23. Scatter diagram demonstrating the relationship between the ADP disaggregation ratio and the response to adrenaline in mm.

n = 21 r = 0.68 p =< 0.001


100
80
60
40
20
0
% DISAGGREGATION
>100
100
80
60
40
20
0
RESPONSE TO ADRENALINE IN MM

The results and statistical analyses are tabulated in Table 6.

3.3.3 ADP disaggregation patterns after the administration of ASA. Ten subjects showing an abnormal response to ADP were tested after an interval of three daystogether with the same number of controls. The results are tabulated in Table 7 and as can be seen, all the results were reproduced.

The abnormal subjects were re-tested 24 h after the administration of two 300 mg tablets of ASA. Every subject involved showed two changes in aggregation response:-

1. No response was observed when collagen was added to the platelets.

2. Every abnormal ADP disaggregation ratio returned to normal. The results are tabulated in Table 8.

3.3.4 Electron microscope studies. Two different preparations were processed for examination under the scanning electron microscope. After the whole blood and the platelet-rich plasma preparations were incubated for 5 mins. on a series of glass coverslips, it was found that the latter gave far superior results when processed and examined under the SEM. Present in these prp specimens were platelets which had exhibited "activity" shown by extrusion of the pseudopodia. Some platelets did not show any activity and appeared as round cells in the preparations. It was also noted that some preparations contained more of the "active" platelets than others. However, when using the method described by Wollweber (210), it was concluded that the preparations were insufficiently washed. This resulted in protein remaining on the coverslips and this was not acceptable in electron microscopy studies. These results are illustrated in Fig. 24. This problem was overcome by modifying the original method by introducing extra washing procedures and also including paraformaldehyde in the method. However, it was noted that the round, less active platelets were washed off the coverslip during this procedure.

Table 6 listing the correlation between the ADP disaggregation results and the response to adrenaline in mm.

Significant correlation was obtained ($p = < 0.001$) when the fall of the adrenaline in mm was compared with the "percentage disaggregation" ($r = 0.68$) and "disaggregation ratio" ($r = 0.83$).

No response to the adrenaline was noted in 50 subjects demonstrating normal platelet disaggregation.

No.	ADP disaggregation		Fall of adrenaline in mms.
	Percentage.	Ratio.	
1	0	150.0	143
2	14.2	7.0	96
3	0	162.0	220
4	0	100.0	67
5	36.6	2.7	38
6	34.2	2.9	60
7	0	80.0	110
8	0	40.0	56
9	43.6	2.3	40
10	30.3	3.3	54
11	0	150.0	110
12	14.2	7.0	86
13	0	86.0	136
14	0	140.0	180
15	0	80.0	100
16	23.3	4.3	60
17	31.0	3.2	52
18	43.4	2.3	29
19	25.0	4.0	40
20	16.3	6.1	82
21	0	40.0	60
Mean	14.8	51.1	86.6
Standard Deviation	15.9	57.6	48.4

Correlation (r) between the "percentage disaggregation" and the fall in adrenaline = 0.68.

$t = 4.1$ $p < .001$

Correlation (r) between "disaggregation ratio" and the fall in adrenaline = 0.83.

$t = 6.5$ $p < .001$

Table 7 showing the ADP disaggregation ratio results after an interval of 3 days.

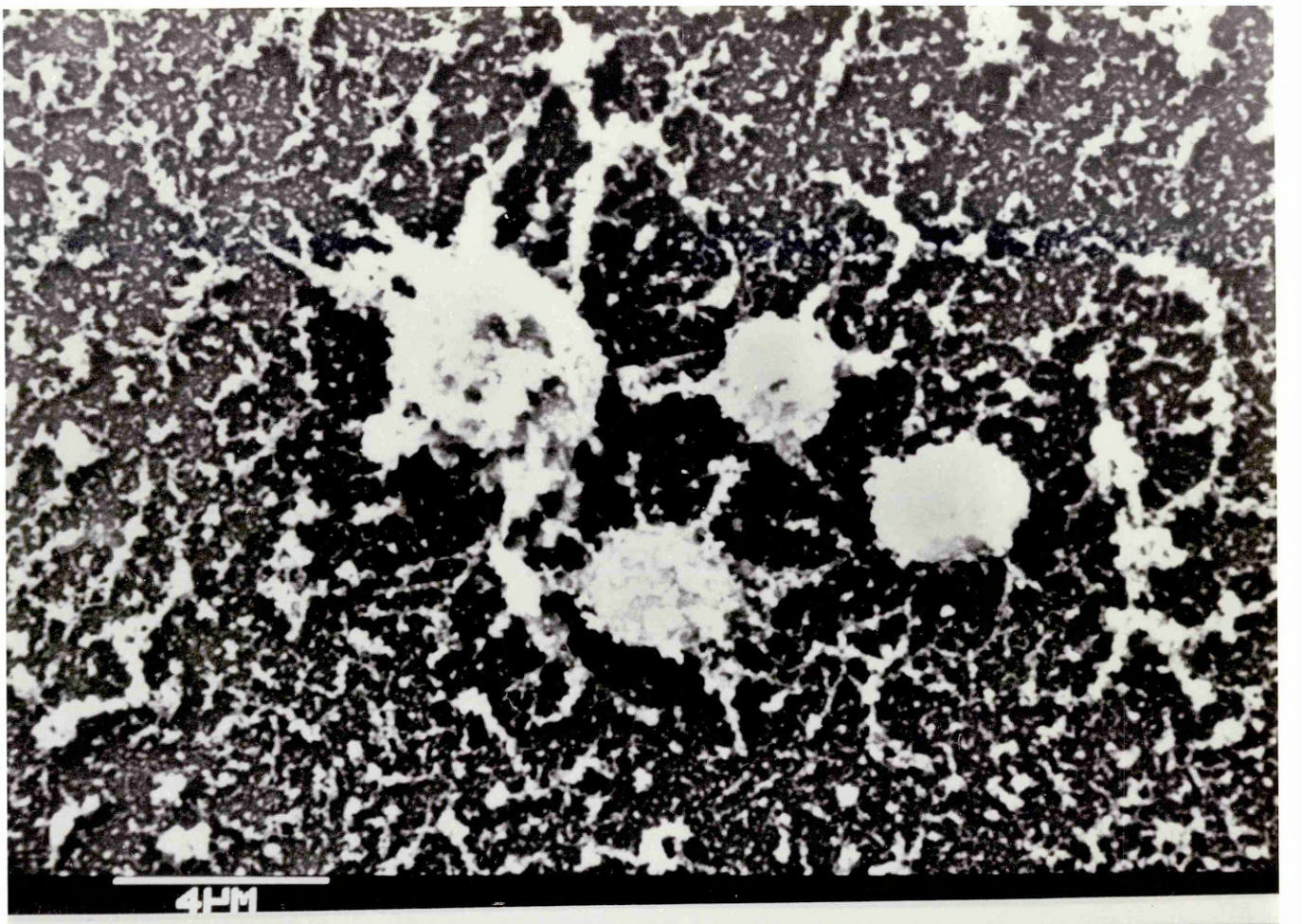
Included are 10 normal and abnormal results.

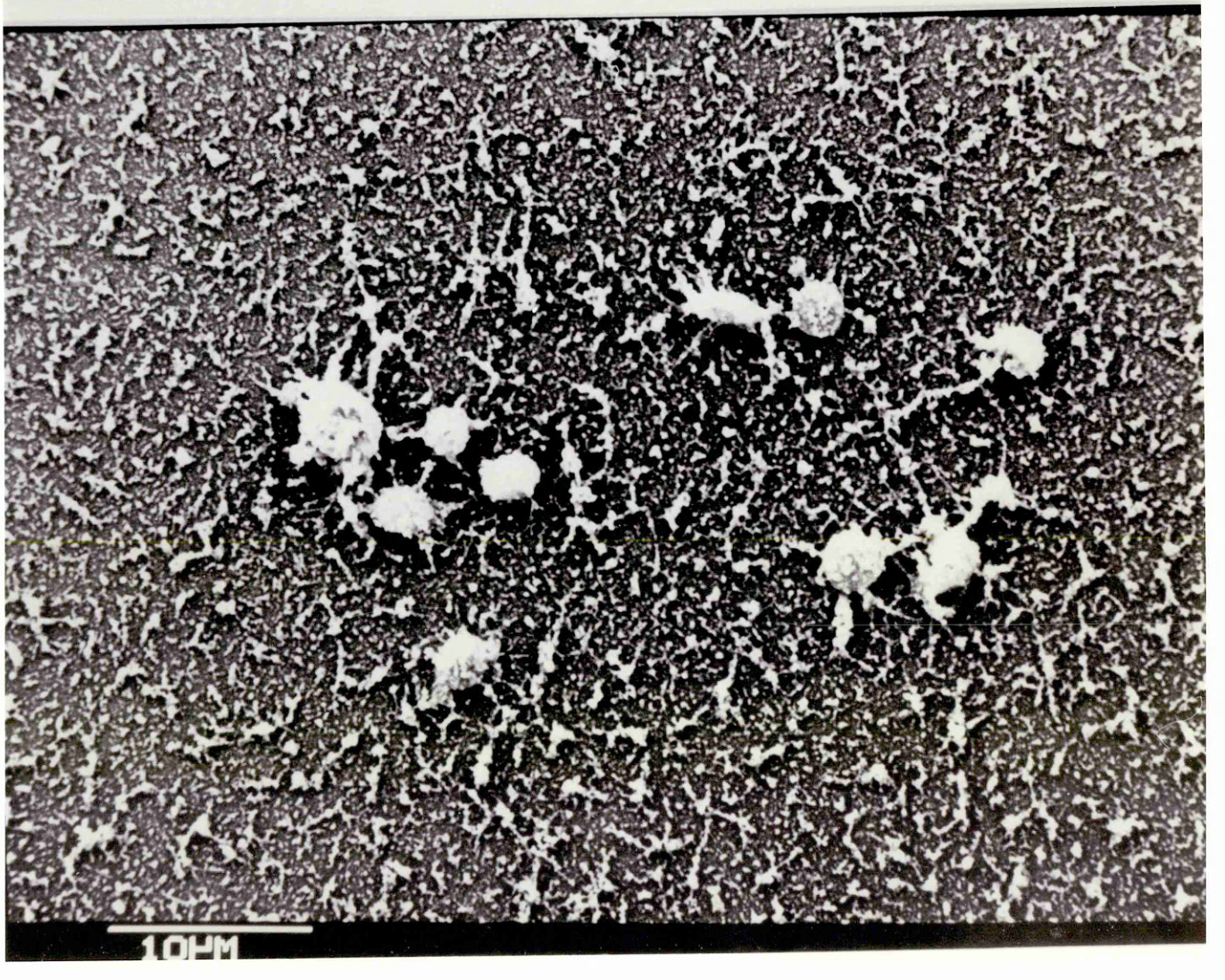
Patient	ADP Disaggregation Results Day 1	ADP Disaggregation Results After an Interval of 3 Days
1	36.0	29.2
2	4.0	3.5
3	100.0	74.0
4	26.2	23.0
5	5.3	4.2
6	26.1	18.2
7	4.1	3.4
8	100.0	92.0
9	28.2	24.1
10	2.3	2.1
11	1.1	1.4
12	1.8	1.8
13	1.9	1.6
14	1.3	1.3
15	1.9	1.7
16	1.8	1.9
17	1.7	1.4
18	1.6	1.4
19	1.8	1.6
20	1.9	1.9

Table 8 showing the ADP disaggregation ratio results after the administration of ASA.

	ADP Disaggregation Results Day 1	ADP Disaggregation Results After an Interval of 3 Days	ADP Disaggregation Results After The Administration of ASA
1	36.0	29.2	1.9
2	4.0	3.5	1.8
3	100.0	74.0	1.9
4	26.2	23.0	1.6
5	5.3	4.2	1.4
6	26.1	18.2	1.4
7	4.1	3.4	1.6
8	100.0	92.0	1.9
9	28.2	24.1	1.7
10	2.3	2.1	1.5

Fig. 24 illustrating the insufficient washing of the platelet preparation when using the method described by Wollweber (210).


4μM


1.0μM

The problem was overcome by activating all the platelet population present in the preparation. This was achieved by increasing the time that the platelets were in contact with the glass and ensuring it was sufficient for the less "active" platelets to change shape and adhere to the coverslip. Further work showed that the modified washing procedure did not remove platelets from the coverslip if the incubation time was increased to 10 mins. Identical activity results were obtained with incubation times of 15, 20 and 30 mins. although these were unsatisfactory because of the drying of the preparations during incubation resulting in further problems with protein precipitation.

The original method of examination after 5 mins. incubation was repeated in parallel with the modified method after 10 mins. incubation. Examination of the SEM preparations showed that the platelet-rich plasma specimens showed the same total number of cells present in both tests. No cells were lost. In this modified preparation, with 10 mins. incubation, the round cells had changed shape, and although not extruding many pseudopodia, had clearly adhered to the surface of the glass coverslips.

The platelet activity demonstrated by direct examination using the scanning electron microscope is illustrated in Figs. 25-27. The results are compared with the aggregation response after the addition of a "critical dose" of ADP.

Figure 25 shows a normal ADP disaggregation response and as can be seen the activated platelets were separate and no aggregation was observed on the SEM preparation.

Figure 26 was from a community based MI patient who had suffered infarction at least 12 months previously. The ADP disaggregation demonstrated an abnormal pattern and the SEM preparation showed the platelets grouped in aggregates of five to ten.

Figure 27 illustrates the grossly abnormal ADP disaggregation normally found in acute hospital based MI cases.

Fig. 25 showing a normal ADP disaggregation response. The activated platelets are separate and no aggregation can be observed.

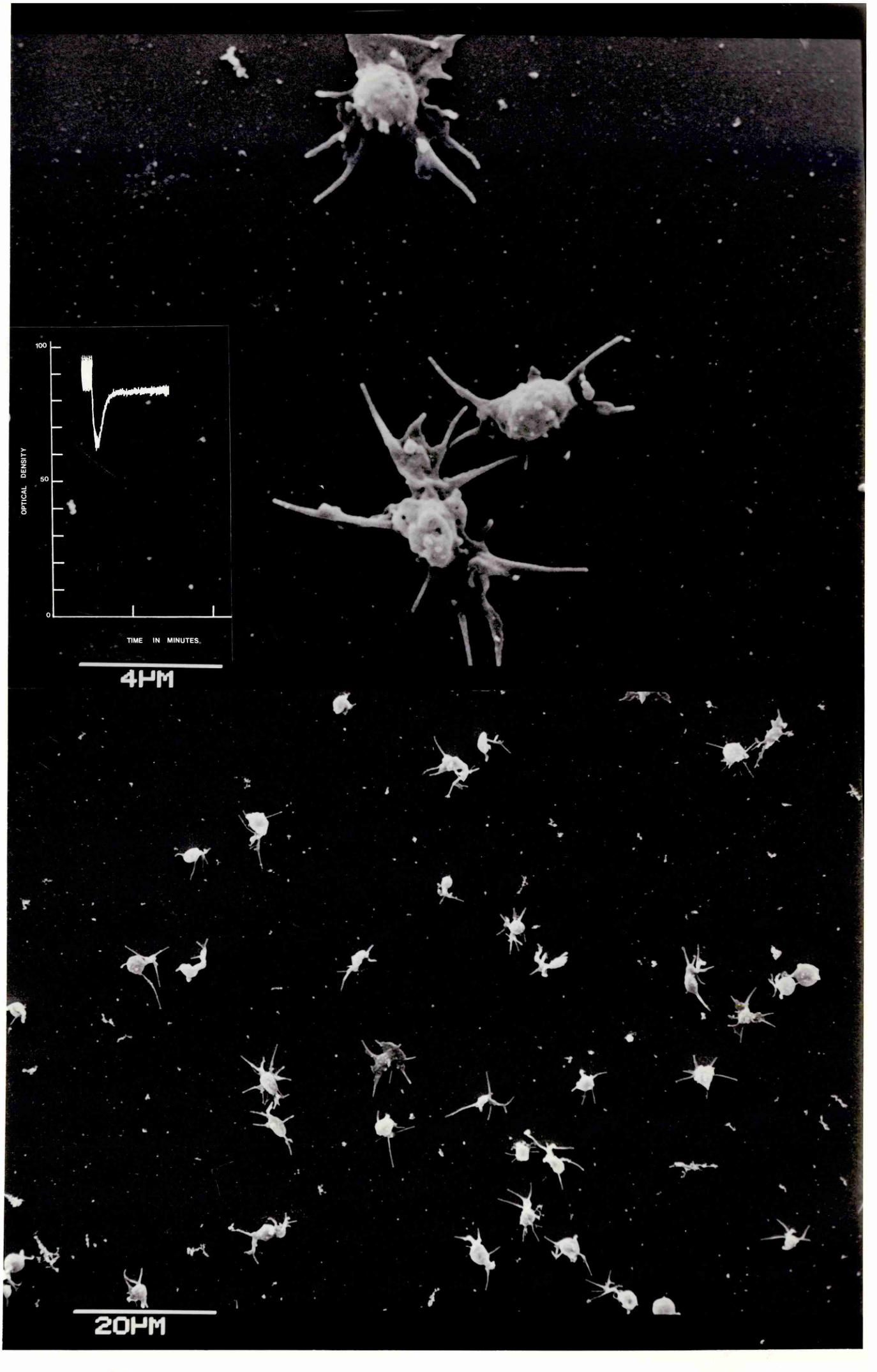
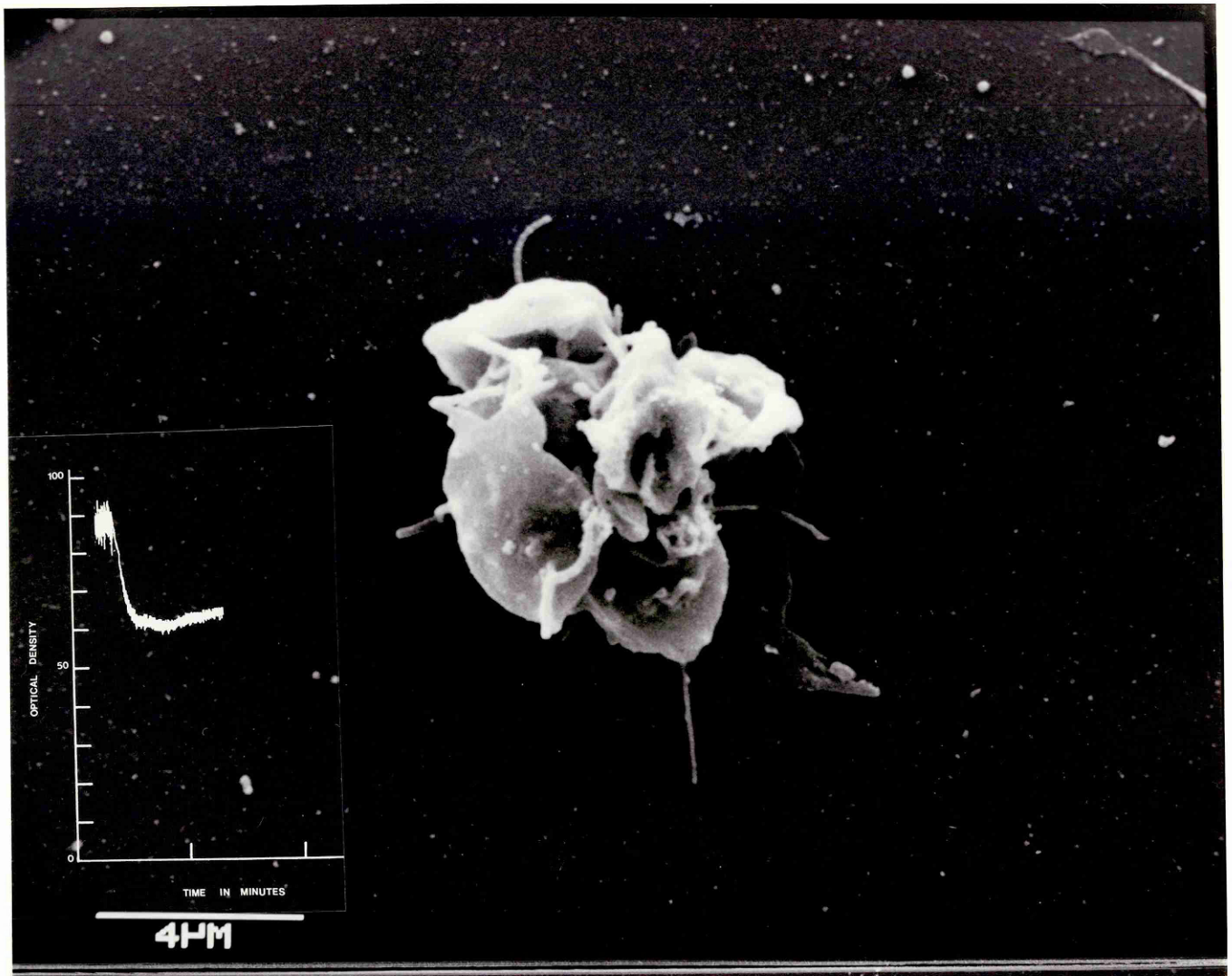
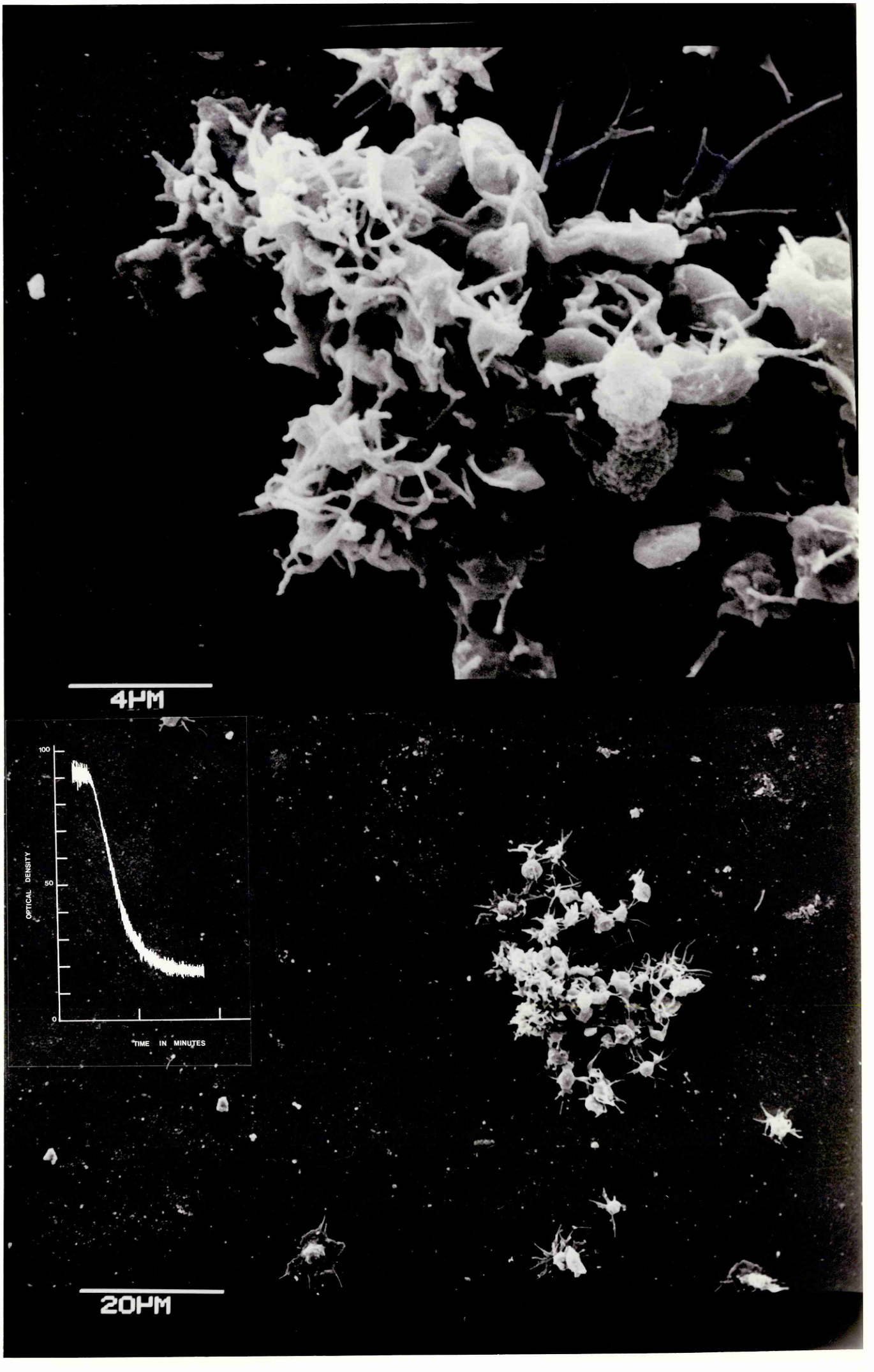

100
50
0
OPTICAL DENSITY
TIME IN MINUTES.
4HM
20HM

Fig. 26 showing an abnormal ADP response in the "community" based MI patient. The activated platelets are grouped in aggregates of 5-10 cells.


100
50
0
OPTICAL DENSITY
TIME IN MINUTES
4μM


20μM

Fig. 27 showing the grossly abnormal ADP response seen in acute cases of MI. The activated platelets are grouped in aggregates of 10-20 cells.


4μM
100
50
0
OPTICAL DENSITY
TIME IN MINUTES
20μM

As can be seen the platelet preparation demonstrates platelets grouped in aggregates of ten to twenty.

All the platelets in every preparation tested had changed shape and adhered to the glass. In the specimens where the ADP disaggregation patterns were abnormal gross fusion of the pseudopodia were more prominent in the SEM preparation. This experiment was repeated using twenty different populations of platelets and similar results to those described above were obtained.

3.4 Discussion

This chapter describes an experimental programme designed to investigate different aspects of the ADP disaggregation test developed and described in the previous chapter.

The EEL system used was compared with a basically different system of measuring platelet aggregation. Page 38 lists the important differences between the ADG and the EEL aggregation meters. Each one of these factors could affect the aggregation and subsequent disaggregation of the test sample. The results show that a satisfactory correlation was obtained between the two systems used.

The disaggregation patterns obtained after the addition of a "critical dose" of ADP were compared with the response in the same platelet population to adrenaline. As discussed on page 45, correlation was obtained between the activity observed when the platelets were challenged with the two aggregating agents. Every preparation that showed an abnormal disaggregation response to the ADP also showed a response to adrenaline. Where there was no response in the latter, the disaggregation ratios were always normal.

The last two experiments performed investigated the hypothesis that:-

1. The abnormal aggregation patterns were due to the platelets

releasing their endogenous ADP from the dense granules.

2. The abnormal results reflected the presence of an excessive number of "active" platelets in the circulation of such patients.

The first hypothesis (1) was investigated by administrating ASA to those patients who exhibited abnormal disaggregation patterns. This drug has the effect of preventing the release reaction of the dense granules by interfering with the formation of thromboxane A_2. (This process has been detailed on page 28). Therefore, after ASA has been administered to such patients, the disaggregation patterns should return to normal if the abnormal results were due to the release of endogenous ADP. Every platelet-rich plasma prepared 24 h after ASA, showed a return to normal disaggregation. These results support the hypothesis that the abnormal disaggregation ratios were due to the release of the dense granules containing the endogenous ADP.

The second hypothesis (2), that the blood of subjects exhibiting an abnormal disaggregation pattern contained an increased percentage of "active" platelets was investigated using electron microscopy. It was demonstrated that an increased activity was observed when the platelet ADP disaggregation ratios were abnormal. Such responses have been demonstrated in Figs. 25-27.

The investigations confirm that the abnormal disaggregation tests reflected the abnormal platelet activity in the patients' circulation due to an excess of "active" platelets. The methods described involved "indirect" tests using aggregating agents, response to ADP after the administration of ASA and "direct" examination of platelet activity under the scanning electron microscopy after activation with glass.

CHAPTER 4

ADP DISAGGREGATION AND COMMUNITY STUDIES

4.1 Introduction

This chapter describes various experiments performed in an attempt to find another test reflecting the same platelet abnormalities as demonstrated by the disaggregation patterns. This was with the aim of performing large scale field work in conjunction with the Medical Research Council Epidemiology Unit.

Preliminary development work showed that the only difficulty most likely to be encountered with the ADP aggregation test would be the time factor between venepuncture and testing. Specimens tested immediately and retested at time intervals up to six hours after bleeding gave satisfactory reproducibility. Results were unreliable if the specimens were left longer. This limited the number of tests that was acceptable to the laboratory during the working day, additionally the MRC was restricted to studies close to Carmarthen. This necessitated the development of a method suitable for large scale community work in the field.

It was considered that this problem could be overcome in two ways:-

1. The technology could be transferrred to the MRC centre where the community study was being undertaken and the tests performed immediately. However, ADP is unstable when made up in solution and requires storage at critical temperatures. The adrenaline solution described on page 41 could overcome the storage difficulties as this reagent is purchased in sealed vials in a liquid form and is stable at room temperature. This adrenaline test could be used as a complete test with the fall in aggregation representing the platelet abnormality. Alternatively, it could be used as an initial screening test, following

which, the patients with an abnormal response would be requested to attend the Carmarthen laboratory for the ADP disaggregation test.

2. A new test capable of being performed in the base laboratory could be developed. Certain criteria would need to be met by such a new test.

It must correlate with the ADP disaggregation test and be similarly capable of demonstrating abnormal results in a percentage of "normal" controls and the community based MI subjects. The ultimate specimen prepared should be stable and therefore suitable for posting to the base laboratory. In addition, it would be advantageous if the test could be automated with capability of assaying the large number of specimens expected when performing large scale community studies. As the platelet is unstable during storage it was decided to concentrate this investigation on parameters consequent upon platelet aggregation and measurable in plasma. Two such factors were considered; platelet factor 4 and β-thromboglobulin.

Platelet factor 4 is a small molecular weight protein that is secreted from the α-granules of platelets during the release reaction (211-213). This action is referred to as "type II release" and has been detailed on page 6. This phenomenon occurs when the platelets become activated subsequent to contact with subendothelial tissues and a variety of other physiological agents including thrombin, ADP and epinephrine (212). These have been listed in Table 1. Nath and his coworkers (214) describe PF4 as a protein that inactivates heparin activity. It has been suggested that increased levels of platelet factor 4 (PF_4) in the blood could serve as an estimate of intravascular platelet aggregation (214). This factor is well suited for inclusion in this investigation because raised levels have been reported in thrombotic states and also in myocardial infarction (215-220). It is known that other

proteins (221, 222) can neutralise heparin and O'Brien (215) refers to this PF_4 test as "heparin-thrombin clotting time" (HTCT). However Nath and his co-workers (214) conclude that "PF_4 antigen and antiheparin activity are two properties of the same protein".

β-Thromboglobulin was isolated and characterised by Moore and his co-workers (223). This factor is located in the α-granules of platelets and is freed when platelets undergo their release reaction (224). It can be considered a platelet specific protein, the presence of which is an expression of the platelet release reaction. As with PF_4, an increase is present in the blood of MI patients (225). Because of these considerations it has been included in the investigations.

4.2 Materials and Methods

4.2.1 ADP disaggregation and β-Thromboglobulin levels. The β-Thromboglobulin assay has been performed using a radioimmune-assay (RIA) kit available from The Radio-chemical Centre, Amersham, Bucks. The RIA method is dependent on competition between β-Thromboglobulin and ^{125}I labelled β-Thromboglobulin for a limited number of binding sites on a β-Thromboglobulin specific antibody. The amount of ^{125}I-labelled β-Thromboglobulin bound by the antibody is inversely proportional to the concentration of unlabelled β-Thromboglobulin present in the platelet-poor plasma samples. The antibody bound ^{125}I-labelled β-Thromboglobulin was separated by precipitation with an ammonium sulphate solution. After centrifugation and removal of the supernatant, the precipitated radio activity was measured in a gamma counter. By measuring the proportion of ^{125}I labelled β-Thromboglobulin bound in the presence of a series of standards, the concentration of β-Thromboglobulin in the unknown samples could be calculated from a standard curve. In addition, the system employed a mixture of anti-coagulant and anti-platelet reagent which, combined with

maintaining the blood sample at 4°C, prevented the release of β-Thromboglobulin from the platelets during processing. In this way, the measured β-Thromboglobulin reflected the in vivo circulating level at the time of sampling.

The ADP disaggregation tests were performed as previously outlined on page 29. Aliquots of the blood samples were treated by the method described in the RIA kit and the β-Thromboglobulin level assayed.

4.2.2 ADP disaggregation and platelet factor 4. The PF_4 levels were assayed using two different methods. The original work was performed using the "clotting" method developed by Fuster (226) and O'Brien (215). The observations made were confirmed using an RIA method which only became commercially available towards the latter stages of the investigations.

The "clotting" method of assaying PF_4 was basically a very simple test. Use was made of two inactivation processes; PF_4 inactivated heparin and heparin inactivated thrombin. When thrombin was added to plasma, it combined with the fibrinogen to form the fibrin clot. Heparin was added to the test plasma and was inactivated by the PF_4 present. Thrombin was added which was immediately inactivated by the heparin and the thrombin remaining combined with the fibrinogen in the plasma to form the fibrin clot.

The series of reactions were as follows:-

Plasma (PF_4)	Heparin inactivated according to the level of PF_4 present in the plasma.
+	
Heparin	
+	Heparin remaining after the above reaction inactivated the thrombin.
Thrombin	

+

Fibrinogen in plasma

+

Fibrin clot

The thrombin remaining after the above reaction combined with the clotting factors to form the fibrin clot.

The more PF_4 that was present in the plasma the more heparin that was inactivated. Therefore less thrombin was neutralised and more was left to combine with the fibrinogen in the plasma and the fibrin clot formed in a shorter time.

When normal or reduced amounts of PF_4 were present, less heparin was neutralised and more was left to inactivate the thrombin. More thrombin was therefore inactivated and less was left to combine with the fibrinogen and the fibrin clot took longer to form.

The clotting time after the addition of thrombin therefore reflected the level of PF_4 present in the plasma. The test as described by O'Brien (215) involved adding 0.1 ml of heparin to 0.1 ml plasma, this was left for 1 min and 0.1 ml thrombin was added. The clotting time was measured and recorded. The method described by Fuster (226) was based on the same principle but the coagulation factors were removed from the patients' plasma specimens by incubation at 60°C for 10 mins. The clotting factors for the formation of the fibrin clot were made available from pooled normal "substrate" plasma added for this purpose. Both tests were found suitable for use but the total volume of 0.3 ml in the O'Brien method, compared with 0.7 ml in the Fuster method, made the former the method of choice. The main reason for this decision was that the relatively small total volume involved made it practicable to use the Fibrometer (Beckton-Dickinson, Cavley, Oxford) for the detection of fibrin clot. This is a very sensitive electronic method of measuring clot formation and is used routinely in hospital laboratories for coagulation studies. The thrombin was used at a concentration of 10 units/ml and the heparin was adjusted with every batch of specimens tested until the clotting

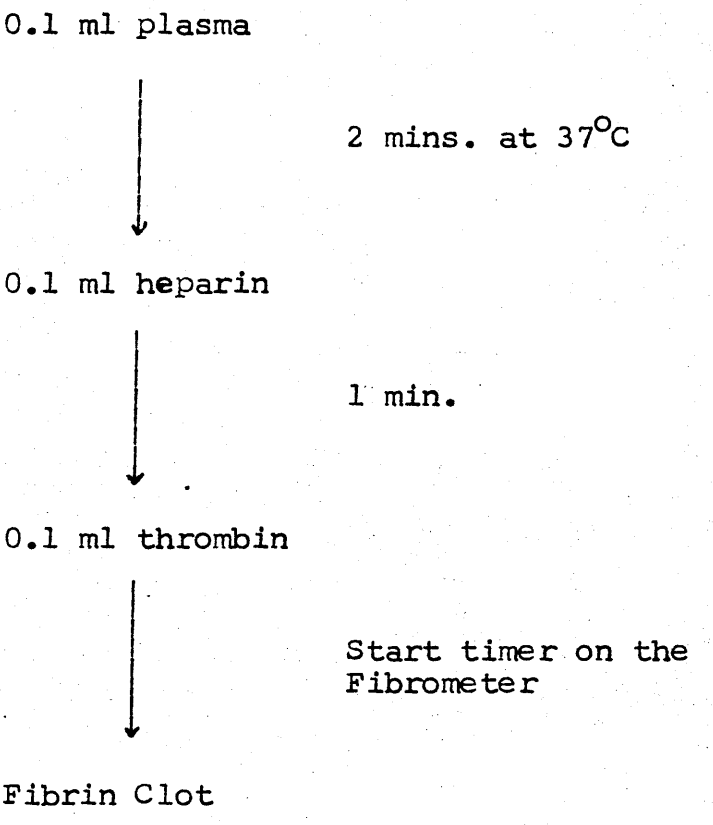
time after the addition of thrombin was between 30 and 40 secs. with "normal" plasma.

Many difficulties were encountered during preparatory work and the original method was modified. These aspects are discussed in greater detail on page 57. Blood specimens were obtained from normal healthy subjects, community and hospital based MI patients. A 9 ml volume of the patients' blood was prevented from clotting with 1 ml 3.13% (w/v) sodium citrate. This was mixed by inversion and, using a plastic pipette, 2 ml was added to each of two plain plastic tubes. One tube was centrifuged immediately at 2400Xg for 15 mins. and 0.5 ml of plasma removed without delay from the top portion of the specimen. This was done in order to minimise contamination from the centrifuged platelet layer. The remaining tube was left at room temperature for 30 mins., centrifuged and treated as described for the first tube. The 6 ml of blood remaining in the original specimen tube was centrifuged and the ADP disaggregation test performed as previously described on page 28.

The plasma specimens were placed at 4°C immediately after preparation when using the "clotting" technique and at -20°C for the isotope method. Both the thrombin and heparin solutions were diluted in veronal-acetate-hydrochloric acid buffer (pH 7.4). A working dilution of 10 units/ml of thrombin was made and divided into 1 ml aliquots and stored at -20°C. Heparin was "titrated" against normal plasma until suitable results of between 30-40 secs. were obtained. The correct "working" dilution was stored at -20°C but the titration was repeated with every batch of specimens tested. The method is outlined below and it is essential that the incubation times stated are followed.

The stored heparin and thrombin were placed at 37°C for 10 mins. before the first specimen was tested and then they were only used for a further 15 mins. The plasma specimens were brought to room temperature and maintained at 37°C for exactly two minutes

before adding the heparin. The system is illustrated below:-



In addition to the PF_4 test, the thrombin time was also performed on every specimen tested. This test checks the reaction between the thrombin and the coagulation factors in the plasma without interference from the heparin. It was performed using the same method described for the PF_4 but the buffer is substituted for the heparin.

One disadvantage of this test was that it was crucial to control the time and the temperature at which the reagents were tested. Efforts were made to overcome these variables and make the test acceptable for field work involving large numbers of specimens by using totally automated coagulation systems. The specimens and reagents on most of these instruments were stored in a 4°C compartment and transferred automatically to cups maintained at 37°C. Therefore the time and temperature variables were strictly controlled and every specimen including the normal controls for the titration of the heparin

were treated in exactly the same manner. The following instruments were kindly offered on loan and modified to perform the platelet factor 4 test as previously outlined on page 55. "Auto-Fi" coagulation instrument (supplied by American Hospital Supplies (U.K.) Ltd., Station Road, Didcot, Oxfordshire). "Coag-a-Pet" (supplied by General Diagnostics, Ltd., Eastleigh, Hants.). "Biomatic 2000" coagulometer (supplied by Walter Sorstedt, (U.K.) Ltd., Beaumont Leyes, Leicester).

The RIA method of assaying PF_4 was performed on a kit available from Abbott Laboratories (Abbot Laboratories Ltd., Brighton Hill, Basingstoke, Hants.). Again the RIA method was a "competitive" technique in which the non-radioactive PF_4 in plasma competed with a constant amount of ^{125}I-PF_4 for binding sites on a limited amount of PF_4 antiserum. Thus the percentage of radioactive PF_4 bound to the antiserum was inversely proportional to the concentration of non-radioactive PF_4 in the specimen. The antiserum bound (both radioactive and non-radioactive) was separated by precipitation with ammonium sulphate solution. The radio-activity of this complex was measured with a well type gamma scintillation counter. The concentration of PF_4 in the specimen was determined by comparison with a group of standards containing measured amounts of non-radioactive PF_4.

4.3 Results

4.3.1 Relationship between disaggregation ratio and β-Thromboglobulin levels. The comparison between the ADP platelet disaggregation ratios and the β-Thromboglobulin levels in ng/ml is illustrated in the scatter diagram (Fig. 28) and the results tabulated in Table 9. Statistical analyses show no correlation ($r = 0.23$) and the probability (p) was less than 0.2 and greater than 0.1.

4.3.2 Relationship between disaggregation ratio and PF_4. Many problems were encountered with the "clotting" method of

Fig. 28. Scatter diagram demonstrating the relationship between the ADP "disaggregation ratio" and the β- Thromboglobulin level in ng/ml. Statistical analyses show a lack of correlation between these two parameters $r = 0.23$ $p = <0.2>0.1$ $n = 33$.

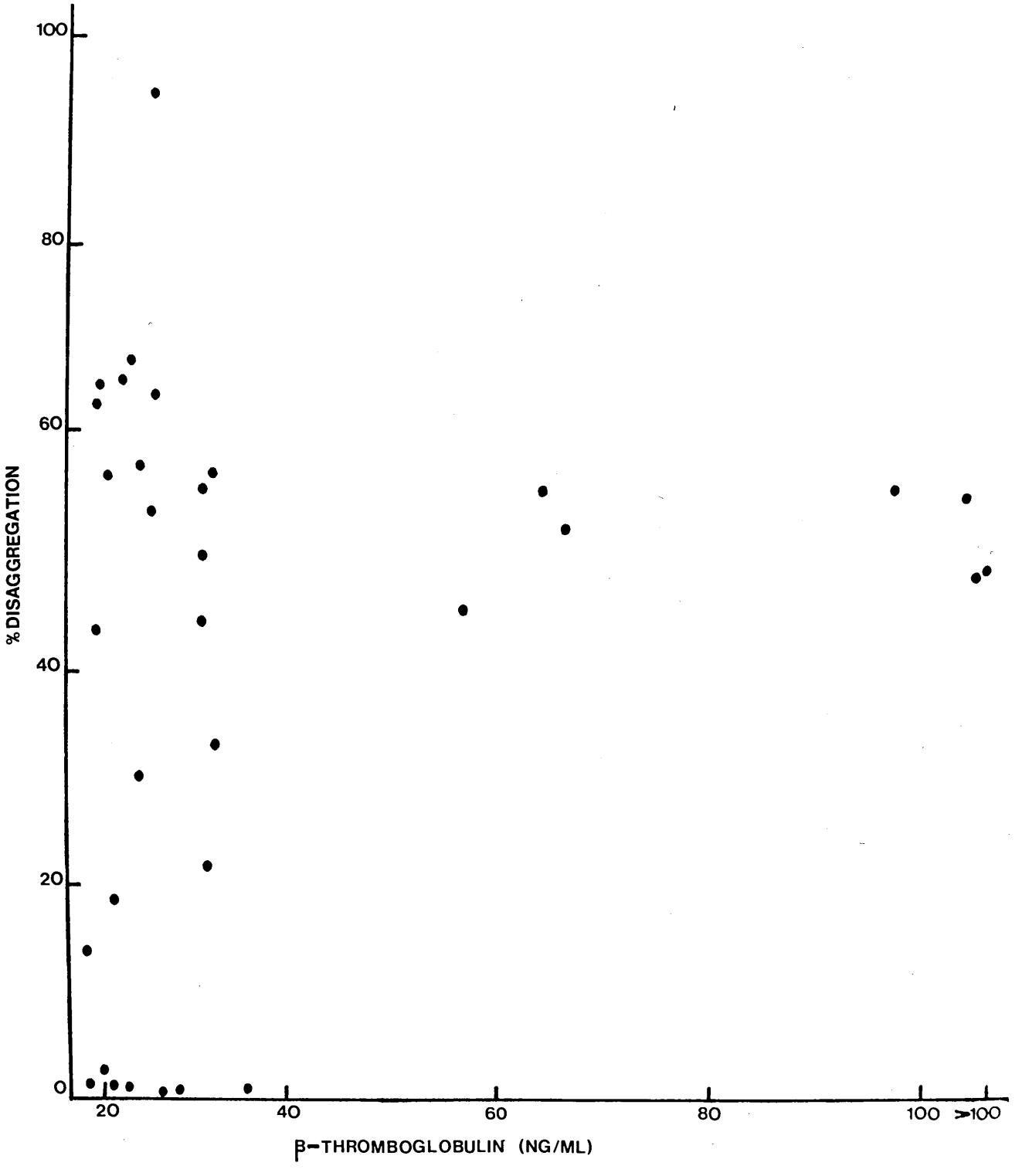

100
80
60
40
20
0
% DISAGGREGATION
20
40
60
80
100
>100
β-THROMBOGLOBULIN (NG/ML)

Table 9. listing the results obtained when comparing the disaggregation results and the β-Thromboglobulin levels in ng/ml.

A lack of correlation was observed ($p = <0.2 > 0.1$) when the β-Thromboglobulin results were compared with the "disaggregation ratio" ($r = 0.23$) and "percentage disaggregation" ($r = 0.24$).

	Disaggregation Results		β-Thromboglobulin levels
	Ratio.	Percentage.	in ng/ml.
1	5.3	19.0	21.4
2	1.6	61.5	19.7
3	30.0	0	21.2
4	1.6	64.0	22.9
5	1.8	54.0	25.9
6	1.8	56.3	33.3
7	29.0	0	38.0
8	10.8	92.3	26.0
9	1.8	56.3	19.8
10	2.3	44.4	32.0
11	1.8	55.2	68.2
12	2.1	49.7	140.0
13	2.2	44.7	58.0
14	1.7	57.6	20.3
15	1.7	57.1	63.0
16	6.9	14.5	19.6
17	1.9	48.7	120.0
18	1.7	58.3	24.3
19	32.0	0	27.6
20	3.3	30.7	34.0
21	14.3	69.8	26.2
22	2.0	50.0	32.0
23	1.8	55.5	120.0
24	2.3	42.9	19.8
25	3.5	29.0	24.6
26	25.0	0	18.2
27	1.8	56.5	98.2
28	68.0	0	28.2
29	1.6	62.1	26.9
30	1.8	57.1	34.2
31	150.0	0	21.6
32	44.0	22.5	32.0
33	160.0	0	18.9
Mean	18.7	36.7	40.5
Standard Deviation	37.7	25.0	32.0

Correlation (r) between "disaggregation ratio" and the β-Thromboglobulin levels = 0.23 t = 1.35 p = $<0.2>0.1$.

Correlation (r) between "percentage disaggregation and the β- Thromboglobulin levels = 0.24 t = 1.4 p = $<0.2>0.1$.

performing the platelet factor 4 test. It was found that the time and temperature at which the reagents were stored and tested were critical. This problem was overcome by strictly controlling these variables and the sensitivity was also improved by the use of the veronal acetate-hydrochloric acid buffer as the diluent. A further change to the PF_4 test was also introduced. This involved the testing of the blood specimen twice. One specimen was prepared immediately after bleeding the patient and the other aliquot of blood was prepared after 30 mins. incubation at room temperature. This modification resulted in a completely new aspect being introduced into the PF_4 test and was developed as the result of problems encountered when standardising the heparin solution to obtain a result of between 30-40 secs. with normal plasma. It was observed that, after the heparin concentration was adjusted to give a satisfactory clotting time with one normal plasma, the next normal tested could vary from a normal to a very abnormal result. Further work showed that these results on the normal plasma specimens were related to the time the bloods were left at room temperature before centrifuging. Typical examples of the results obtained with the different blood specimens separated into 2 ml amounts, and these aliquots centrifuged at controlled time intervals, are illustrated in Fig. 29. It was observed that some blood specimens, although showing a normal result with the sample centrifuged immediately after bleeding the patient, showed a marked increase (decrease in clotting time) in the subsequent samples left at room temperature. Provisional investigations showed that the difference was maximal after 30 mins. It was noted that, although this increase could be demonstrated in the PF_4 test, the thrombin times on all these samples from the same patient, remained the same. The specimens were numbered $P_{(1)}$ (centrifuged immediately) and $P_{(2)}$ (centrifuged after 30 mins.). Similarly the thrombin times on the same specimens were numbered $T_{(1)}$ and $T_{(2)}$.

No correlation was observed when comparing the ADP disaggregation ratio and the platelet factor 4 levels ($P_{(1)}$). Statistical analyses are listed in Table 10 and $n = 41$ $r = 0.26$ $p = <0.1>0.05$. The scatter-diagram is illustrated in Fig. 30.

Fig. 29 demonstrating the difference in the PF_4 levels after different time intervals. One blood specimen was separated into eleven different tubes and each sample centrifuged at the time intervals shown on the chart. Patient X was a normal control and the difference in the PF_4 levels between this specimen and confirmed MI patients Y and Z after 30 mins. incubation can be clearly seen. The normal control and confirmed MI patients demonstrate the same PF_4 levels after an interval of 3-4 h.

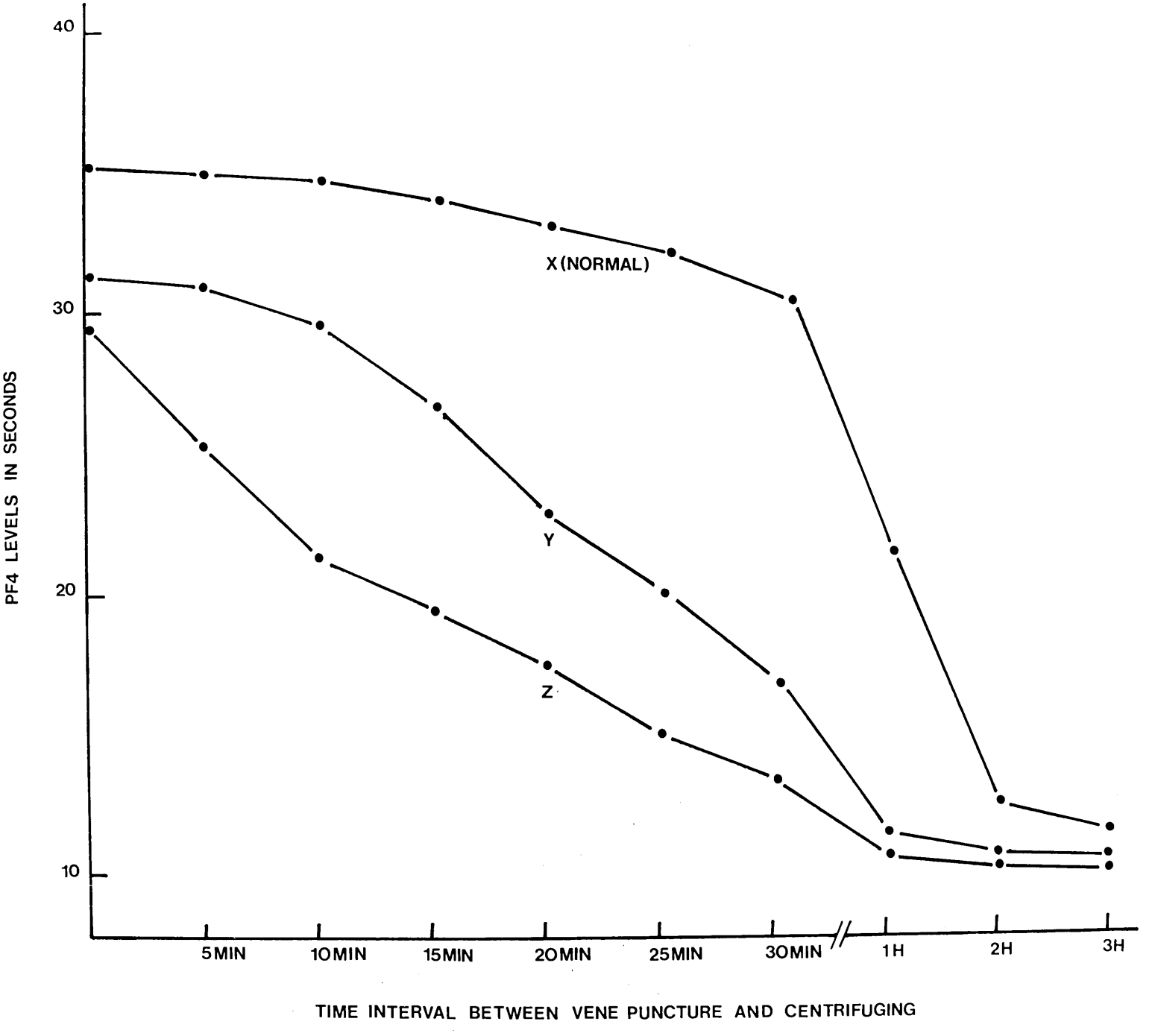

40
30
20
10
PF4 LEVELS IN SECONDS
X (NORMAL)
Y
Z
5MIN
10MIN
15MIN
20MIN
25MIN
30MIN
1H
2H
3H
TIME INTERVAL BETWEEN VENE PUNCTURE AND CENTRIFUGING

Table 10 listing the results obtained when comparing the disaggregation results with the "clotting" method of estimating PF_4.

A lack of correlation was observed when the PF_4 levels were compared with the "disaggregation ratio" ($p = <0.1> 0.05$ $r = 0.26$) and the "percentage disaggregation" ($p = >0.9$ $r = 0.02$).

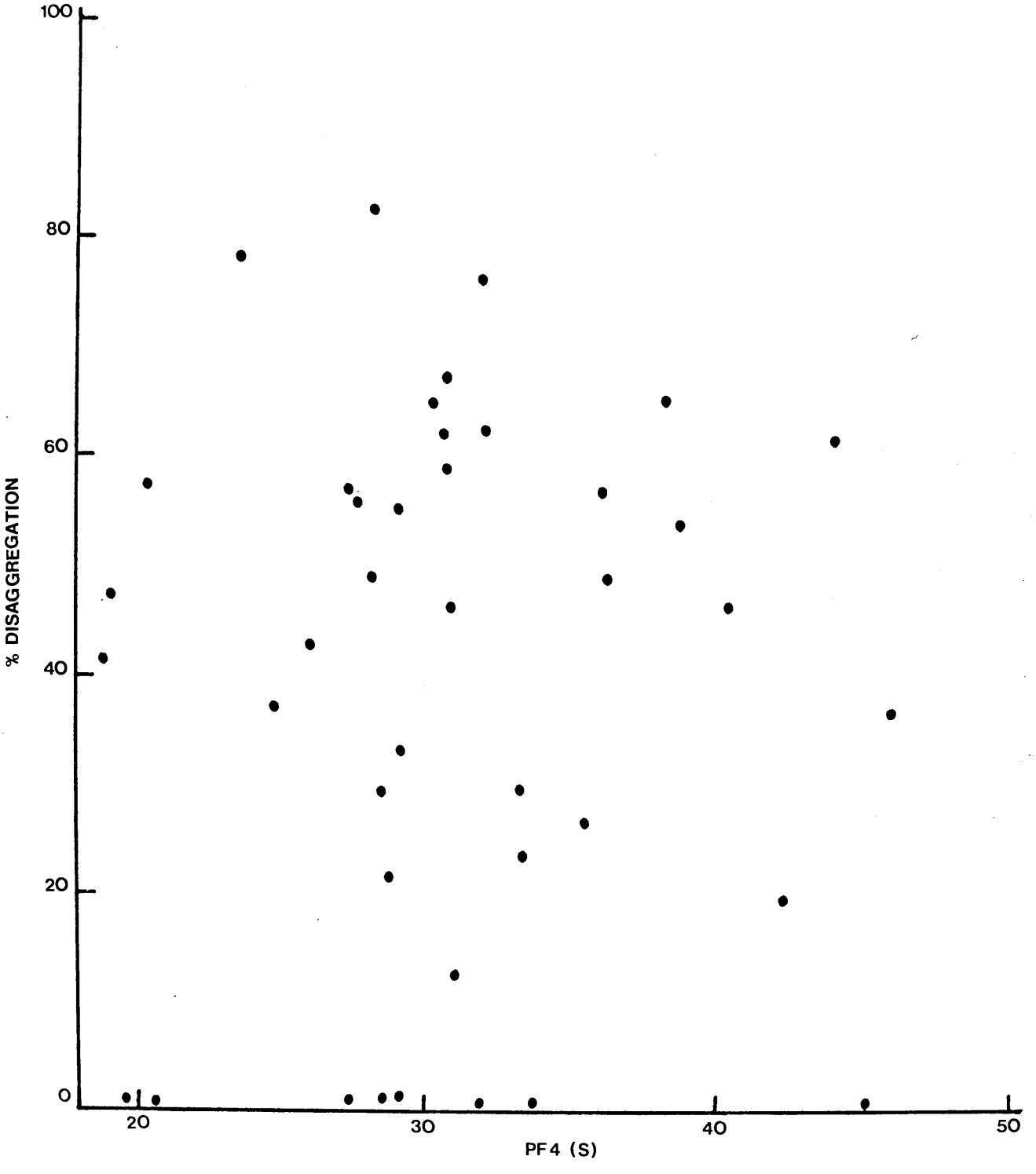
	Disaggregation Results		PF_4 results in secs.
	Ratio	Percentage	
1	1.8	56.5	27.3
2	44.5	22.5	34.4
3	1.7	57.8	20.2
4	2.3	44.4	40.4
5	1.6	64.0	30.2
6	5.3	19.0	43.2
7	3.5	29.0	29.4
8	1.7	57.6	36.2
9	1.7	57.1	27.3
10	1.8	55.5	29.4
11	32.0	0	34.8
12	44.0	22.5	29.4
13	1.6	62.1	30.8
14	32.0	0	45.2
15	160.0	0	21.2
16	1.9	48.7	15.2
17	1.9	46.2	30.4
18	2.3	42.9	26.3
19	118.0	0	15.3
20	38.0	0	34.4
21	1.2	83.3	28.2
22	1.6	63.8	38.4
23	42.0	0	29.3
24	2.1	47.6	28.2
25	1.6	60.5	32.4
26	2.8	35.4	46.2
27	1.7	59.4	30.2
28	182.0	0	29.4
29	2.4	41.4	18.5
30	91.0	0	27.5
31	2.6	38.3	25.0
32	3.4	29.0	33.0
33	1.9	48.8	37.6
34	1.4	60.3	44.0
35	1.8	53.6	39.4
36	1.3	76.9	32.0
37	6.4	15.5	31.8
38	1.2	78.0	24.0
39	4.2	23.8	35.9
40	3.3	30.3	29.2
41	1.5	66.6	31.0
Mean	20.8	38.9	31.0
Standard Deviation	42.0	24.7	7.24

Correlation (r) between the "disaggregation ratio" and the PF_4 levels = 0.26 t = 1.7 p = < 0.1> 0.05.

Correlation (r) between the "percentage disaggregation" and the PF_4 levels = 0.02 t = 0.12 p =>0.9.

Fig. 30 Scatter diagram illustrating the relationship between the ADP disaggregation patterns and the PF_4 level in seconds.

Statistical analyses show a lack of correlation
$r = 0.26$ $p = <0.1>0.05$ $n = 41$


100
80
60
40
20
0
% DISAGGREGATION
20
30
40
50
PF4 (S)

However, examination of the data revealed that 7 out of a total number of 10 acute hospital based MI patients showed abnormally high (shorter clotting time) PF_4 levels. No abnormal results were seen in any of the normal controls or the "community based" MI patients.

When the disaggregation ratio results were compared with the $P_{①}$ and the $P_{②}$ values from the same patients, the difference between these two latter tests could be related to the disaggregation patterns. Three examples of such results are illustrated in Fig. 31. These three patterns show the importance of the modified PF_4 test:-

Pattern (a) was from a normal control and the $P_{①}$ value was normal and the $P_{②}$ result showed a reduction (increase in PF_4) of only 1.8 secs.

Pattern (b) was from a community based confirmed MI patient, the ADP disaggregation pattern was abnormal, the P_1 value normal, but there was a marked reduction in the clotting time of $P_{②}$ (a decrease of 14.3 secs.).

Pattern (c) was from a hospital based acute MI patient and both the aggregation and $P_{①}$ levels were abnormal.

The stringent criteria related to time and temperature introduced to improve reproducibility resulted in the problem that only a few duplicated tests could be performed in one batch. Attempts to automate the test using three different automatic coagulation methods were not successful. Not one of the three instruments tested was found to be as sensitive as the Fibrometer used in the original experiments. It was considered that the reason for this was that the electronic method of detecting the clot was more efficient than the optical density or vibrating rod system used by the automated methods. These methods were not sensitive enough to measure the weak, friable clot formed when the heparin was used at the

Fig. 31 demonstrating examples of the correlation between the ADP disaggregation patterns and the difference between the PF_4 levels in the P_1 and P_2 plasma specimens.

Subject "A" shows a normal ADP disaggregation ratio

The PF_4 results were:-

P_1 = 30.4s

P_2 = 28.6s

The thrombin times were:-

T_1 = 11.2s

T_2 = 11.1s

Subject "B" shows the typical abnormal ADP disaggregation ratio shown by "long term" MI patients

The PF_4 results were:-

P_1 = 32.5s

P_2 = 18.2s

The thrombin times were:-

T_1 = 11.4s

T_2 = 11.4s

Subject "C" was an acute MI patient.

The PF_4 results were:-

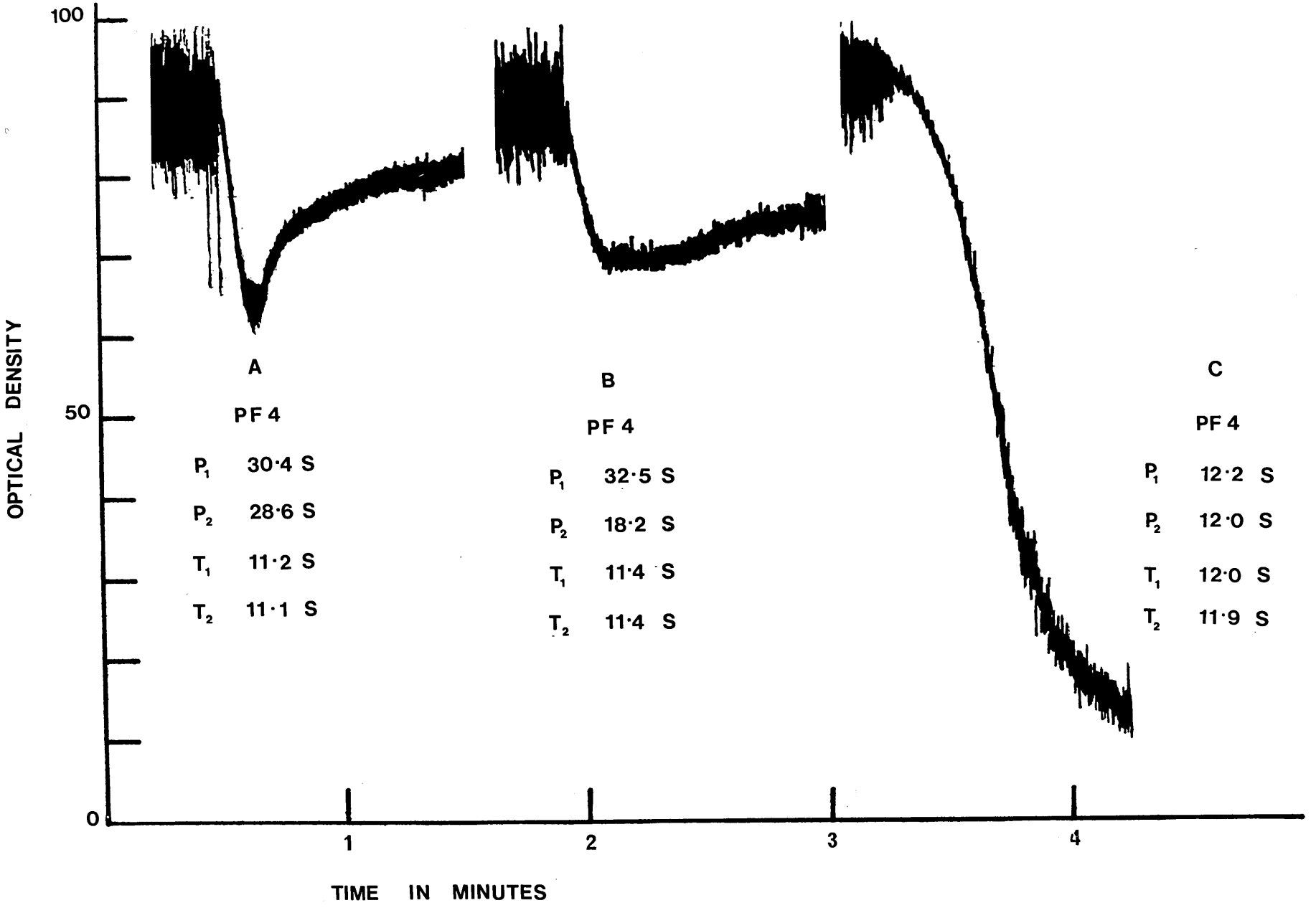
P_1 = 12.2s

P_2 = 12.0s

The thrombin times were:-

T_1 = 12.0s

T_2 = 11.9s


100
50
0
OPTICAL DENSITY
A
PF 4
P1 30·4 S
P2 28·6 S
T1 11·2 S
T2 11·1 S
B
PF 4
P1 32·5 S
P2 18·2 S
T1 11·4 S
T2 11·4 S
C
PF 4
P1 12·2 S
P2 12·0 S
T1 12·0 S
T2 11·9 S
1
2
3
4
TIME IN MINUTES

same concentration as was prepared and titrated on the Fibrometer. However, they functioned satisfactorily when the heparin concentration was reduced. This resulted in the formation of a sturdier fibrin clot but the differences between the $P_{①}$ and $P_{②}$ values were not as great as found with the Fibrometer. The same loss of sensitivity was encountered when the tests were performed manually. In addition the results could not be reproduced the following day because of the loss of labile clotting factors which affected the formation of the fibrin clot.

The modified platelet factor 4 test incorporating $P_{①}$ and $P_{②}$ differences not only detected abnormalities in the acute MI cases but was capable of showing an abnormality in the community based MI patients and also in a percentage of normal control subjects. These abnormal groups showed a normal $P_{①}$ value but the abnormality was detected in the increase seen after incubation at room temperature for 30 mins. ($P_{②}$). Over 80 subjects have been tested comprising of community based normal and confirmed MI patients. Examples of the results obtained are illustrated in Table 11.

The platelet factor 4 RIA method was also performed. Preliminary tests showed that plasma specimens required dilution 1:3 with the diluent provided in the kit. This facilitated the reading of the test result from the most sensitive portion of the "standard graph". The graph and some of the results obtained can be seen in Fig.32 and examples of the results obtained are listed in Table 12. It can be seen that this method confirms that marked differences in the $P_{①}$ and $P_{②}$ results exist in some of the subjects tested. Only limited data has been obtained to date from experimental work incorporating this method. However, it appears that a similar significant relationship between the disaggregation ratio and the platelet factor 4 levels in the $P_{①}$ and $P_{②}$ specimens described using the clotting method can be reproduced using the RIA technique.

Table 11 showing examples of the ADP disaggregation patterns and the platelet factor 4 levels after centrifuging immediately and after incubation at room temperature for 30 mins.

The PF_4 levels are reported in seconds. The pairs of results indicate the P_1 and P_2 values and the thrombin times are similarly reported. (A) denotes abnormal platelet disaggregation results and (N) a normal response.

Platelet disaggregation ratio	PF_4 levels reported in seconds	Thrombin times reported in seconds
2.6 (A)	25.0 16.0	12.0 11.9
3.4 (A)	33.0 20.0	11.7 11.6
1.9 (N)	37.6 34.0	11.8 11.8
1.4 (N)	44.0 41.0	11.7 11.8
1.8 (N)	39.4 34.0	11.6 11.6
1.3 (N)	32.0 31.0	11.9 11.9
6.4 (A)	31.8 19.0	11.7 11.7
1.2 (N)	24.0 24.0	11.9 11.7
4.2 (A)	35.9 17.3	11.8 11.8
3.3 (A)	29.2 14.0	12.0 11.9
1.5 (N)	31.0 28.2	11.8 11.7
100 (acute MI)	12.0 12.0	11.9 12.0

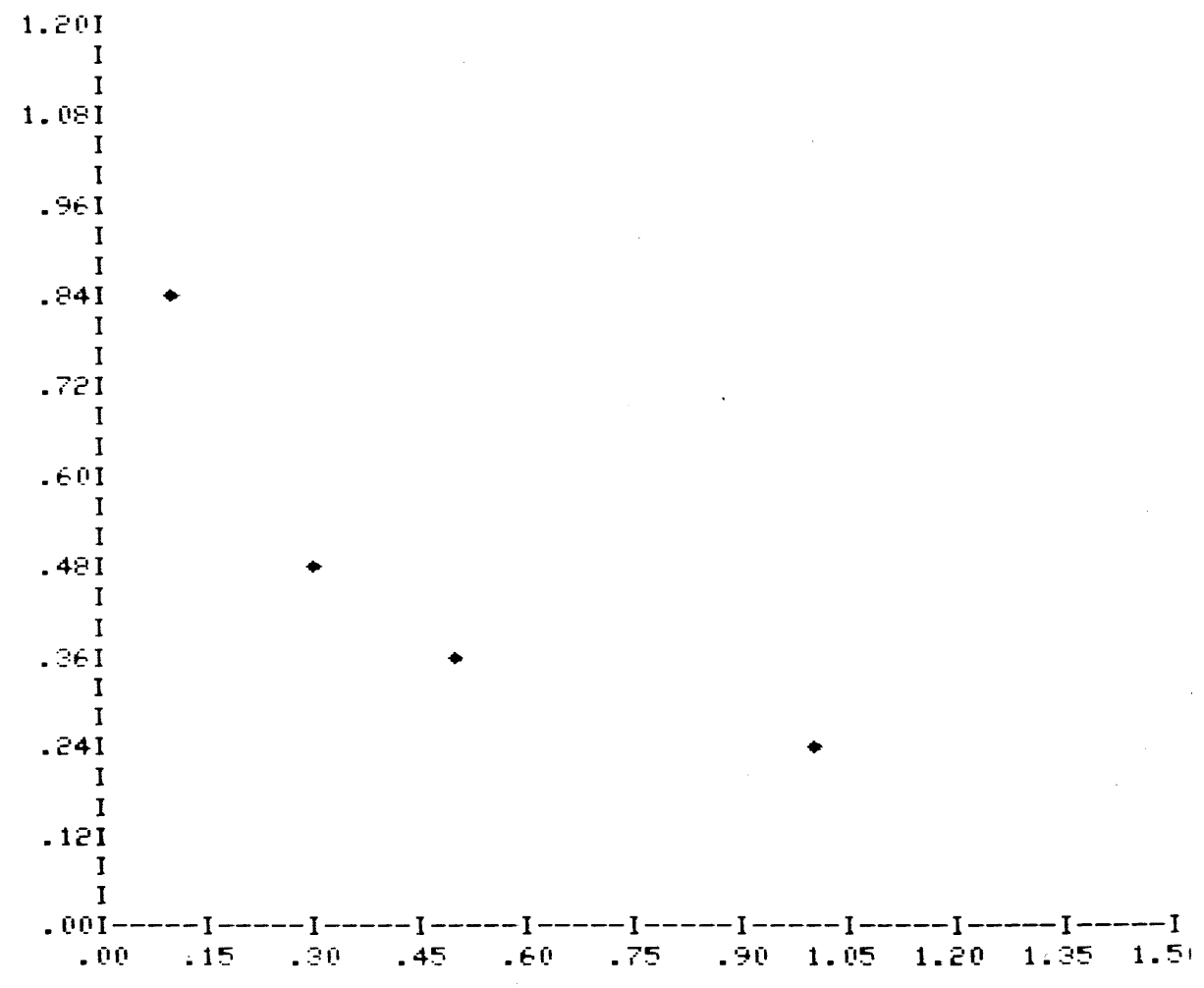
Fig. 32 demonstrating the "standard graph" and examples of the way in which the data was presented. The "conc." are the PF_4 values in ng/ml.

The gamma counter used was an LKB (LKB Instruments Ltd., Addington Rd., Selsdon, Croydon) model "Rockgamma 11". A Texas Instrument (St. James House, Wellington Rd:, North, Stockport) "silent 700 electronic data terminal" was programmed with the appropriate information and the data was presented as shown.

	CPM
BLAN	1993.0
REFR	4672.0
TOTA	14543.0

	CONC	B/B0
STD01	10.000	.842
STD02	30.000	.491
STD03	50.000	.392
STD04	100.000	.256

B/B0 E 00



POS	CODE	TIME	COUNT1	CPM1	%	RATIO	CONC.
0008	UNKS	0060	004082	2089	1.6	.447	38.855
0009	0000	0060	005869	3876	1.3	.830	10.719
0010	0000	0060	004798	2805	1.4	.600	23.772
0011	0000	0060	001932		2.3	.000	194.173!
0012	0000	0060	005875	3882	1.3	.831	10.646
0013	0000	0060	003830	1837	1.6	.393	49.741
0014	0000	0060	001936		2.3	.000	194.173!
0015	0000	0060	003931	1938	1.6	.415	45.378
0016	0000	0060	003537	1544	1.7	.330	72.598
0017	0000	0060	004538	2545	1.5	.545	26.941
0018	0000	0060	002721	728	1.9	.156	136.850!

Table 12 listing examples of the PF_4 RIA results. The data indicate that some of the patients tested showed the significant marked increase between $P_{(1)}$ and $P_{(2)}$ that was observed when using the "clotting" method.

The pairs of results describe the $P_{(1)}$ and $P_{(2)}$ values in ng/ml.

Platelet Factor 4 results on P_1 and P_2 specimens reported in ng/ml
10.7
23.7
26.9
220.0
30.4
45.2
18.2
33.0
12.0
24.4
33.7
38.0
20.3
96.4
26.6
283.0
25.7
117.9
26.3
40.1

4.4 Discussion

This chapter describes investigations performed in order to replace the ADP disaggregation patterns with a test suitable for Medical Research Council field work. Investigations were concentrated on the platelet factor 4 and the β-Thromboglobulin levels in the patients' plasma. These factors have been well documented and are known to play a role in the platelet release reaction. The tests available were reliable and capable of investigating large numbers of requests expected from a population survey. Denham and his co-workers (225) showed that β-Thromboglobulin was abnormal in some MI patients and suggested that the results might be of practical value in the care of such patients. Similar results were obtained in this study and abnormal results were found in 6 out of 10 acute MI patients. However, normal values were obtained with the community based "long-term" MI patients and no correlation was found between the ADP disaggregation ratio and the β-Thromboglobulin levels. In the PF_4 studies, 7 out of 10 acute MI cases were abnormal. No correlation was obtained between this test and the ADP disaggregation ratio. Not one of the community based MI patients demonstrated an abnormal PF_4 result. The only test performed that was capable of showing abnormal results in these community based MI patients and showed a correlation with the ADP disaggregation was the modified PF_4 method. This was achieved by using a very sensitive system of detecting the clot formed and also by centrifuging a portion of the blood immediately and after 30 mins. at room temperature. It was found that the disaggregation pattern was related to the difference in the PF_4 level between these two plasma specimens. Pairs of patients' plasma specimens showing abnormal results were fully investigated. Coagulation screenings for levels of clotting factors as well as individual factor assays were performed. No difference in any of the coagulation factors was found in the pairs of plasma specimens tested. It has been reported (221, 222) that other proteins can inactivate heparin. The utilisation of two specimens from the same patient excludes this phenomenon as the explanation for the results obtained; proteins should show no increase in

concentration during incubation at room temperature for 30 mins. Therefore, the only possible source of this increase in PF_4 levels must be attributed to the platelets. It was demonstrated that a good correlation existed between abnormal ADP disaggregation tests and a marked increase in the PF_4 levels. The first tests confirmed that an increased percentage of "active platelets" was present in these subjects and it is suggested that the same phenomenon accounts for the increase in the PF_4 levels. This view is based on the observation that all the blood specimens tested were found to have a normal whole blood platelet count (the majority having a total count within the range 250-350 x 10^9/l). Therefore, the only difference between the normal and abnormal results was that the latter group released in 30 mins. the same level of PF_4 that the normal subjects released in 3-4 h. Therefore, the abnormal group released their platelet factor 4 at a faster rate than the normal subjects. Karpatkin (30, 134) has demonstrated that "active" platelets release PF_4 more readily than the less active cells. It must be assumed that platelets release some of their α-granules during the 30 mins. incubation at room temperature. However, the amount released is relatively small and only detected because of the sensitive techniques used. The PF_4 levels assayed by the RIA method demonstrated an increase from approximately 16 ng/ml ($P_①$) to 40 ng/ml ($P_②$). In comparison, when PF_4 was assayed in serum, results of greater than 400 ng/ml were obtained. The time factor variable (up to six hours) has been shown (227) to have no influence on the ADP disaggregation test.

The modified platelet factor 4 test fulfilled the criteria demanded for a test that could replace the ADP disaggregation patterns. The problem of returning large numbers of blood samples to the base laboratory was therefore overcome. The samples can be centrifuged and stored by the MRC staff and returned to the laboratory for the platelet factor 4 assay using the RIA method.

1. The previous chapters have shown that in the apparently normal healthy male population is a sub-group demonstrating abnormal platelet behaviour when exposed to ADP as shown in disaggregation tracings. These patterns were similar to those found in patients recovered from MI. It was subsequently demonstrated that such abnormal patterns reflected an increased number of "active" platelets present in the circulation of these subjects.

2. The MRC Epidemiology Unit wished to identify individuals in the population "at risk", that is, those most likely to develop thrombosis and coronary heart disease.

3. Platelets cannot be stored and therefore this resulted in technical and field work difficulties.

4. Two different approaches were:-

   a. to consider a test and related apparatus that could be used by the MRC in the field.

   b. to study a stable product in plasma released by the platelets.

5. Work was performed on β-Thromboglobulin and Platelet Factor 4. The primary objective was to determine a parameter suitable for field work giving results correlating with the ADP disaggregation patterns. It was observed that if a specimen of blood was separated into two aliquots, one centrifuged immediately and one after 30 mins. incubation; PF_4 results were obtained which correlated with the ADP disaggregation patterns.

6. It is considered that this represents an advance in both the knowledge of platelet behaviour and the technological methods of studying them. The relevance of the results obtained in relationship to the study of coronary heart disease is discussed in the "General discussion" chapter of this thesis.

CHAPTER 5

THE AUTOMATION OF THE COATED TANNED RED CELL HAEMAGGLUTINATION TECHNIQUE

5.1 Introduction

In addition to the work on platelets already described, this thesis is also concerned with the immunological theory of coronary heart disease and, in particular, immunological explanations of platelet disorders related to this disease. The studies described are based on the work of Davies and his co-workers (178) who found a relationship between antibody levels to whole dried milk and patients with confirmed CHD. This observation initiated the interest and co-operation of the Medical Research Council Epidomiology Unit, Cardiff. The main objective became the testing of this original report using large numbers of MI patients and community "control" subjects from a wide area of South Wales. It became essential that sera be tested as quickly and efficiently as possible maintaining quality control. The method employed in the original work was the manual tanned red cell haemagglutination technique and this chapter describes the automation of this test to meet the MRC requirements.

5.2 Materials and Methods

The technique which was automated was the coated tanned red cell haemagglutination method developed by Boyden (228) and modified by Gunther (172). The antigen which was related to coronary heart disease in the original experiments was a soluble preparation of commercially available whole dried milk. This was therefore the antigen of choice used in developing the automated technique.

5.2.1 Manual Method. The reagents used were:- 0.06M phosphate buffered saline (pH 7.4) (PBS) Stock solution of 1% (w/v) tannic acid in distilled water. The working solution was

prepared by diluting the stock $^{1}/_{150}$ with PBS.

The group O Rhesus positive cells used in this work were preserved and anticoagulated with acid citrate dextrose and obtained from the Blood Transfusion Service.

The principle of the method was that group O cells were "tanned" with tannic acid. Reiner and Fischer (229) describe how tannic acid alters the surface properties of the red cell, changing them from a hydrophilic to a hydrophobic state. This results in the absorption of proteins on to the red cell surface.

Group O Rhesus positive cells from the acid citrate dextrose anticoagulated blood were washed three times in the phosphate buffered saline (PBS). The cells were tanned by adding 0.5 ml of packed cells to 5 ml PBS, mixing and adding a further 5 ml of $^{1}/_{15000}$ dilution of tannic acid. The cells were mixed and left at room temperature for 30 mins. after which they were centrifuged and washed once in PBS. To the washed cells obtained by centrifugation, 5 ml of PBS was added and also an equal volume of the soluble antigen solution, which in this development work was prepared from whole dried milk. This was mixed and left at room temperature for 45 mins. when the cells were centrifuged and washed twice in the PBS. The suspension of the antigen coated cells for the titration were diluted in PBS.

In the titration, serial dilutions of the test sera were made in neutral rabbit serum and an equal volume of the sensitised cells added. The titration trays were left at room temperature for 1 h followed by overnight incubation at 4°C. The results were read by noting the pattern formed by the settled cells (230). Negative results were denoted by a sharp round button of cells at the bottom of the well. In contrast, a positive result showed the cells settled in an uniform pattern covering the base of the well. This is illustrated in Fig. 33.

Fig. 33 demonstrating the cell patterns observed with the tanned red cell haemagglutination technique. The results of the sera demonstrated in the haemagglutination tray illustrated in this figure are listed below. The results are read from right to left and the first row contains sera only to facilitate automatic dilution by the Dinateck system. The results are recorded as the number of wells demonstrating a positive result (see page 67).

Row Number	Result
1	2
2	2
3	6
4	5
5	6
6	5
7	<1
8	<1
9	<1
10	<1
11	2
12	3

The protein solution of whole dried milk was prepared by dissolving 3g of the milk in 15 ml PBS. The suspension was shaken for 10 mins. and centrifuged at 2400Xg for a further 10 mins. The supernatant was carefully removed, care being taken in removing the soluble portion only. The total protein concentration of this soluble whole dried milk was assayed using the method of King and Hazlewood (231). In this method, the protein concentration was proportional to turbidity developed with a 3% solution (w/v) of sulphosalycilic acid in 7% (w/v) sodium sulphate. The protein solution of whole dried milk was adjusted with the PBS to give a protein concentration of 500mg/100ml.

The rabbit sera were prepared by inactivating rabbit serum at 56°C for 30 mins. Washed O Rhesus positive cells were added to the inactivated serum and left for 20 mins. The suspension was centrifuged and a working dilution of 1 ml of serum to 200 ml of PBS prepared.

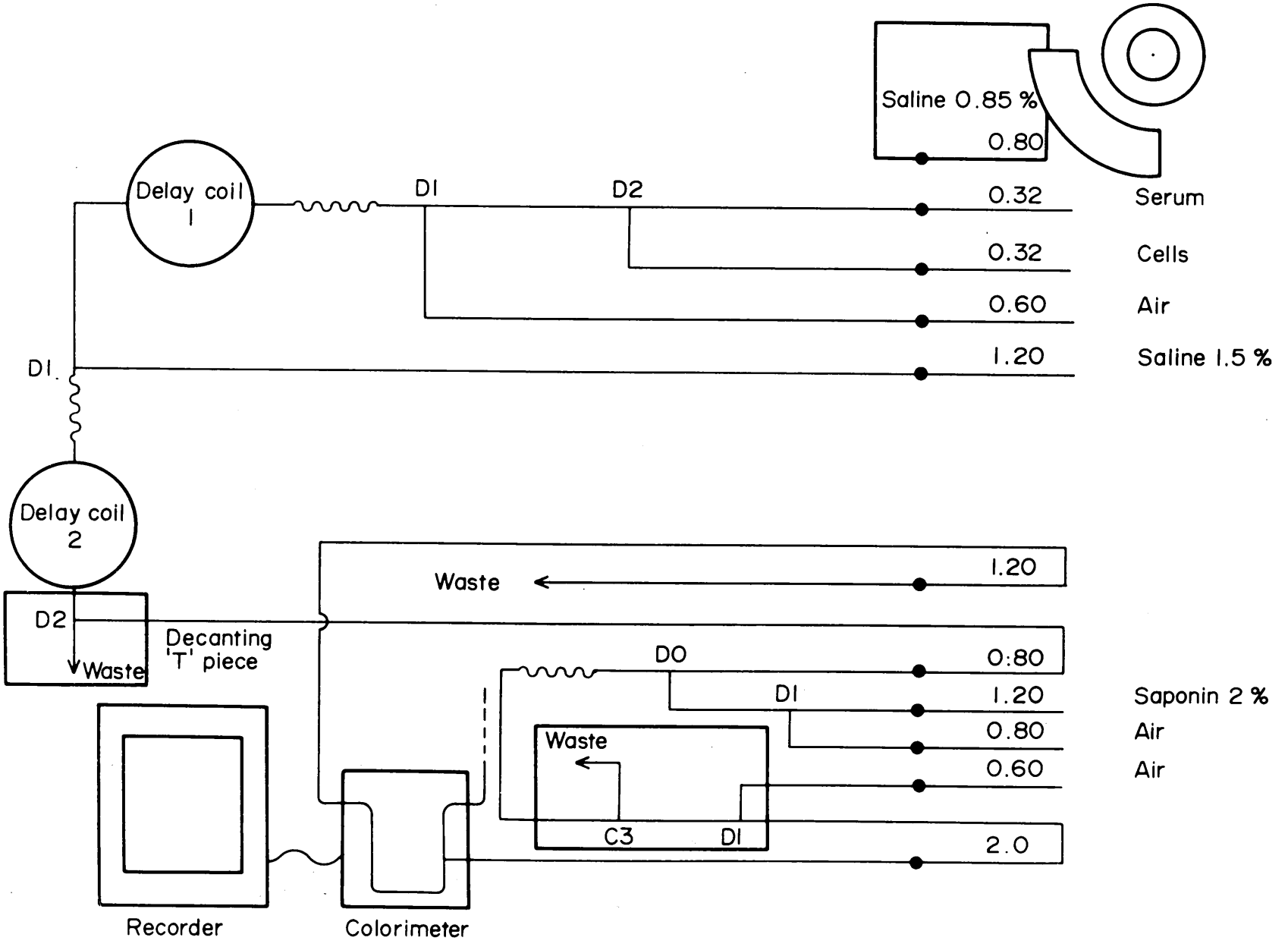
5.2.2 The automation of the coated tanned red cell haemagglutination technique. Cells were tanned and sensitised as described in the manual method. The method was automated using the equipment and tubes available from Technicon, (Technicon, Hamilton Close, Basingstoke). The principle of the method developed was based on agglutination occurring in a glass coil and, using a system of 'T' pieces, these agglutinated cells were removed. After separation from the main "flow", these cells were haemolysed and the colour developed represented the degree of agglutination present in the original specimen. The manifold developed for the automation of the tanned red cell haemagglutination technique is illustrated in Fig. 34.

Using the manifold described, undiluted sera were added to the sensitised cells. After mixing, the antigen/antibody reaction occurred in a 10 mins. glass "time delay coil". A volume of 1.5% (w/v) sodium chloride solution was added after this reaction. This has the effect of destroying any false agglutination caused by abnormal proteins present in the sera

Fig. 34 showing the manifold developed for the automation of the coated tanned red cell haemagglutination technique.

The figures quoted relate to the flow capacity of the tubes used e.g. 0.32 denotes that that size tube was capable of pumping 0.32 ml of fluid per minute.

Reproduced by permission of the publishers.
Rees, B. W. G. (1973) Medical Laboratory Technology, 30, p.168.


Saline 0.85 %
0.80
Delay coil 1
D1
D2
0.32
Serum
0.32
Cells
0.60
Air
D1
1.20
Saline 1.5 %
Delay coil 2
1.20
Waste
D2
Waste
Decanting 'T' piece
D0
0:80
D1
1.20
Saponin 2 %
Waste
0.80
Air
0.60
Air
C3
D1
2.0
Recorder
Colorimeter

(rouleaux formation). The suspension of cells was again mixed and the cells allowed to settle in another coil for 1 min. The agglutinated cells being heavier, settled out faster and were removed by a 'T' piece together with a standard amount of unagglutinated cells. This phenomenon is illustrated in Fig. 35. These decanted agglutinates were haemolysed using 2% (w/v) saponin and the colour developed was measured using a 550 or 535nm filter (See Fig. 36). The peaks observed were therefore in proportion to the colour developed which were directly related to the amount of agglutination present in the specimens.

The detailed procedure is as follows:-

1. Insert 550 nm or 535 nm filter in the colorimeter.

2. Turn on the colorimeter lamp. Allow 20 mins. for lamp to warm up.

3. Place all the reagent lines and sample lines in buffered saline. Start pumping.

4. After 10 mins., check the bubble pattern and connections.

5. Place all reagent lines in their respective reagents. Leave the sample line pick-up in buffered saline.

6. Fill sample cups and place on cover tray to inhibit evaporation. A cup containing buffered saline should be placed between the last standard and the first specimen.

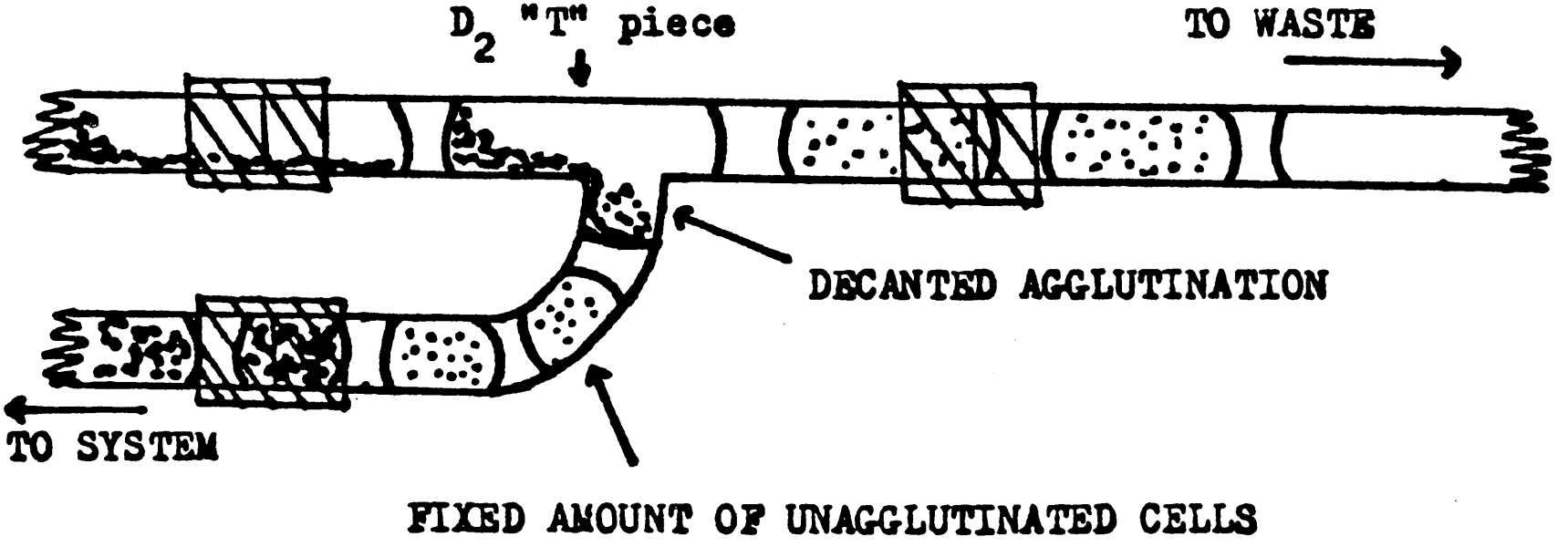
7. When the baseline is established, set recorder at 90-95% transmission (T) also check 0%T and recorder again.

8. Decide on the necessary speed of testing and insert the correct cam.

9. Start the samples and standards through the system.

10. The system should be washed with 1M sodium hydroxide for 5 mins. and distilled water for 25 mins. after testing.

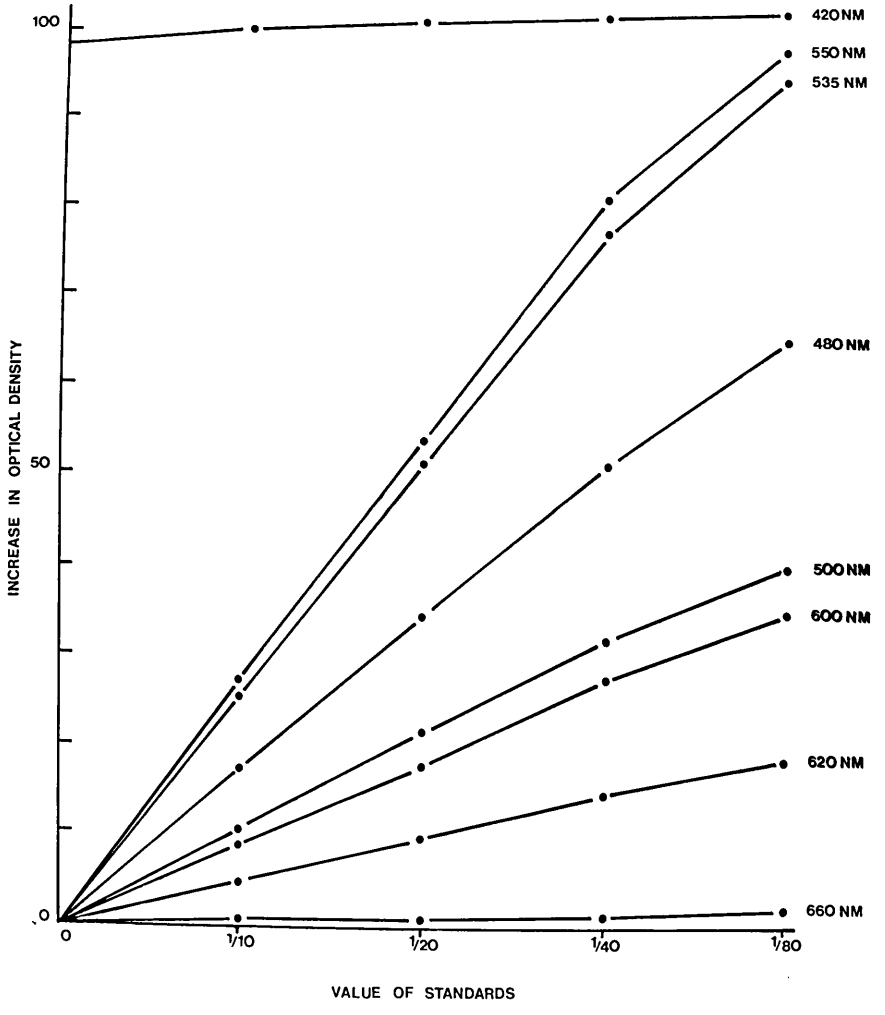
Fig. 35 demonstrating the cells being decanted by the "T" piece. It is important that the "settling out coil" allows the complete sedimentation of the agglutinated cells and leaves the unagglutinated cells in suspension.

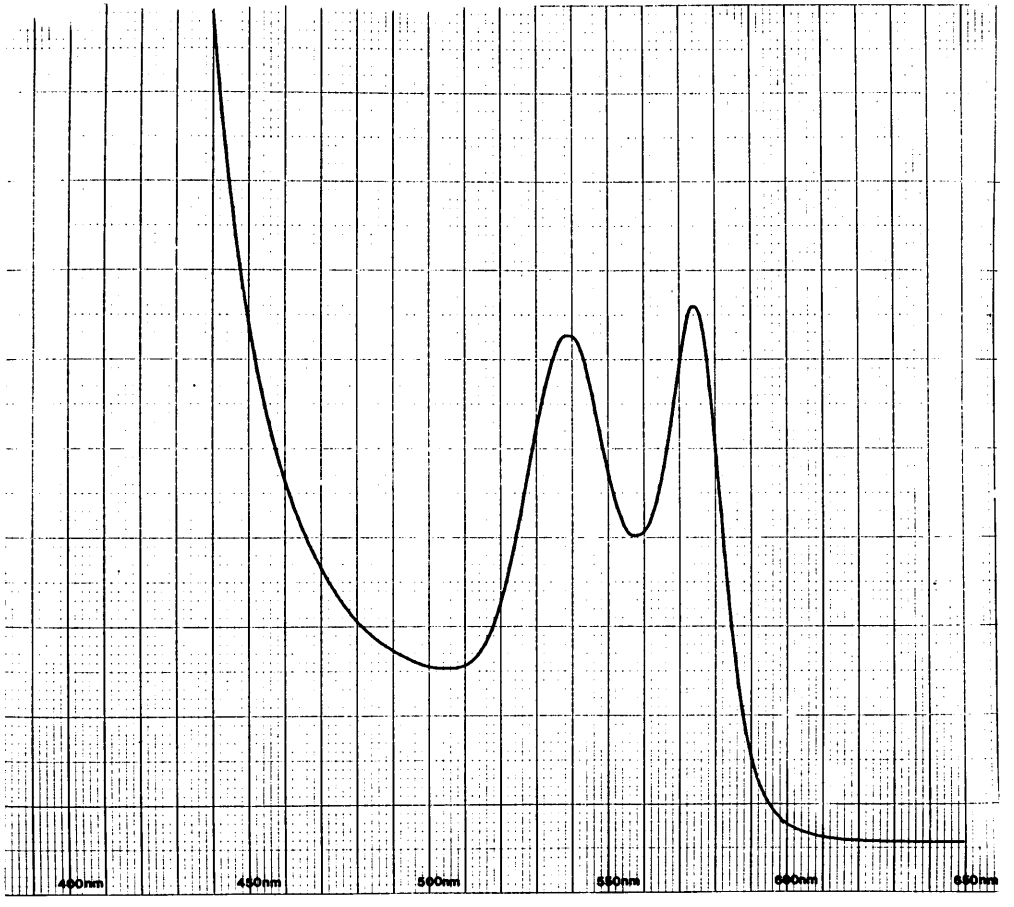

D_2 "T" piece
TO WASTE
DECANTED AGGLUTINATION
TO SYSTEM
FIXED AMOUNT OF UNAGGLUTINATED CELLS

'ig. 36

(Left) A graph demonstrating the peak heights of the standards when different filters were inserted in the colorimeter. Using the 420 nm filter the baseline could not be adjusted to zero.

(Right) A solution of haemolysed cells was scanned on a Pye Unicam "SP8-100 ultraviolet spectrophotometer". The vertical increase reflects the absorbance at the appropriate wavelengths.


100
50
0
INCREASE IN OPTICAL DENSITY
420 NM
550 NM
535 NM
480 NM
500 NM
600 NM
620 NM
660 NM
0
1/10
1/20
1/40
1/80
VALUE OF STANDARDS


400nm
450nm
500nm
550nm
600nm
650nm

5.2.3 Comparison between manual and automated methods. The results obtained using the manual and automated methods were compared by titrating the same sera using the two different techniques. This comparison was performed on relatively small batches at daily intervals. Different preparations of cells were tanned and sensitised on the different days and in addition, varying batches of commercially available dried milk were also purchased and prepared in order to vary the source of the antigenic solutions. A total of 202 sera were tested on different days and all the reagents were changed with each batch titrated.

5.2.4 Recording the results. Using the manual method the sera were double diluted from neat and the results recorded as the number of tubes exhibiting a positive result (i.e. neat = 1 tube, $^{1}/_{10}$ = 2 tubes, $^{1}/_{20}$ = 3 tubes....). When developing the automated technique it was realised that a peak height extending the full length of a chart recorder (i.e. 100% haemolysis or complete agglutination of the serum specimen) was obtained with sera exhibiting an equivalent manual result of $^{1}/_{80}$ (i.e. 5 tubes positive). A batch of sera was therefore titrated manually and all those showing a titre of $^{1}/_{80}$ were pooled and diluted as follows for the automated system:-

Dilution	Titre
Neat	5 tubes ($^{1}/_{80}$)
$^{1}/_{2}$	4 tubes ($^{1}/_{40}$)
$^{1}/_{4}$	3 tubes ($^{1}/_{20}$)
$^{1}/_{8}$	2 tubes ($^{1}/_{10}$)

The "standard graph" was drawn by sampling the above sera first and in addition known "control" specimens were tested every tenth specimen. The peak heights of the unknown sera were compared with the "standard graph" and the results recorded. Sera showing peak heights extending the full length

of the chart were diluted with PBS and the titration repeated.

5.2.5 Additional control tests performed. It was considered that the colour of the sera could influence the resulting peak heights and therefore 60 sera were tested against unsensitised cells. The results would demonstrate the interference in the total peak heights from the colour of the sera.

The reproducibility of the system was tested by titrating the same specimen 50 times and also by repeatedly testing specimens of different titres.

The optimum speed for testing was investigated by titrating 40 specimens manually and repeating the titration using the automated system. The speed of sampling was varied on the automated system from 50 samples per hour to 100 samples per hour.

The optimum protein concentration of the antigenic material was investigated by testing, against 40 sera, the same batch of tanned cells sensitised with varying dilutions of the antigenic material. A similar experiment was performed to find the optimum concentration of sensitised cells suitable for use on the automated system. In this experiment the tanned cells were sensitised with 500 mg/100 ml of the antigenic material and different concentrations of these cells were made and tested in turn against 40 sera.

5.3 Results

Examples of the "peaks" obtained with the automated system are illustrated in Fig. 37. Examples of the comparison between the automated and the manual results are listed in Table 13. The total number of specimens tested in these different batches was 202 and when all the results obtained were compared, the coefficient of correlation r was 0.94 which was highly significant ($p < .001$). These results can be seen in Table 14.

Fig. 37 demonstrating the results obtained using the automated system. Specimens were tested at 60 tests per hour.

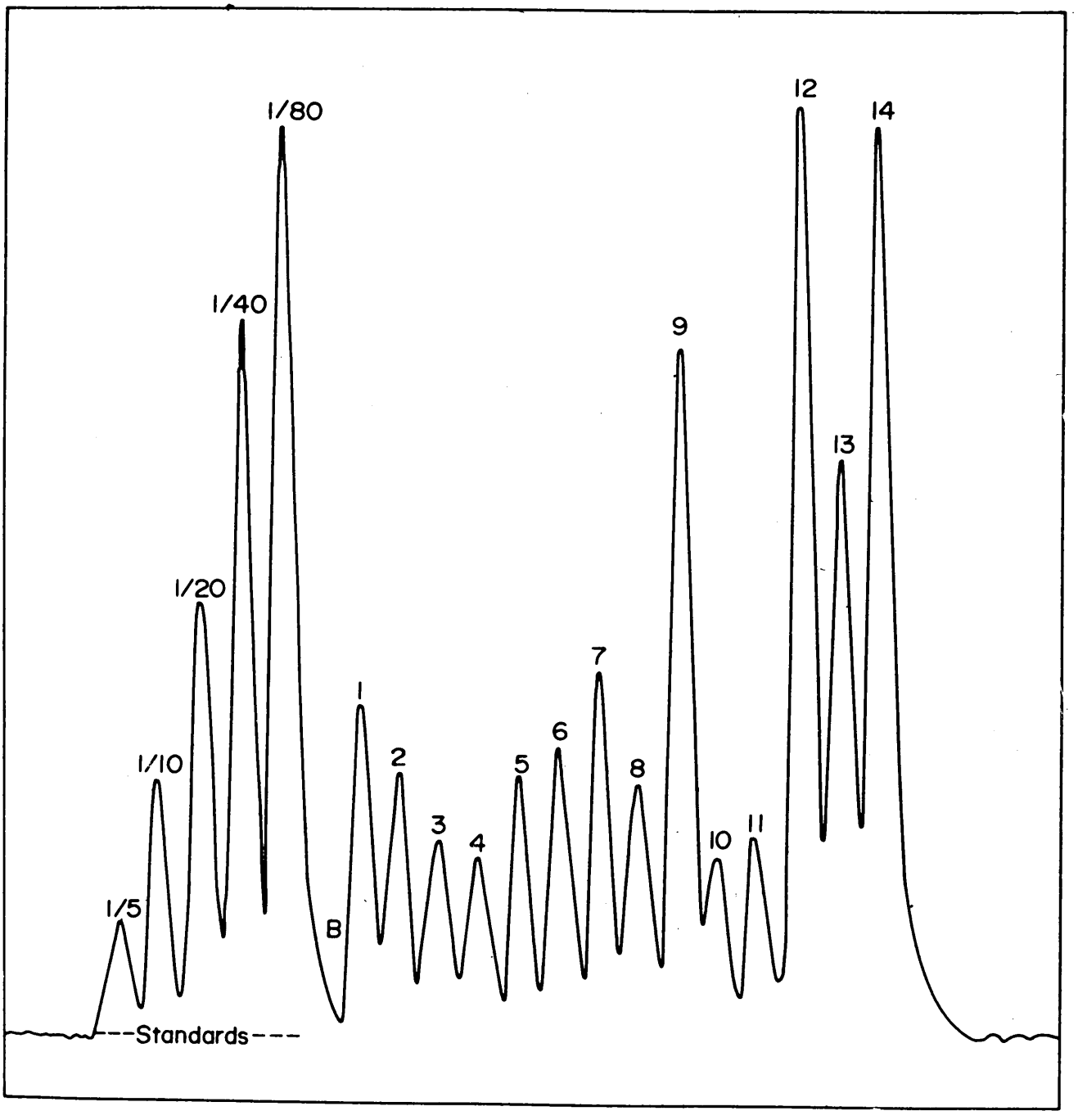

1/80
12
14
1/40
9
13
1/20
7
1
6
1/10
2
5
8
3
4
1/5
B
10
11
---Standards---

Table 13 demonstrating some examples of the comparison between the manual and the automated systems.

Speed 60 Wash 1/2		Speed 70 Wash 1/2	
Manual	Automated	Manual	Automated
8.000	6.000	2.000	2.000
7.000	6.000	3.000	3.000
1.000	2.000	5.000	5.000
7.000	7.000	5.000	3.500
2.000	2.000	5.000	5.000
1.000	2.000	3.000	4.000
6.000	6.000	3.000	3.000
1.000	2.000	2.000	2.000
7.000	5.000	2.000	2.000
3.000	4.000	3.000	2.000
2.000	2.000	2.000	2.000
6.000	6.000	3.000	4.000
3.000	2.000	3.000	5.000
8.000	7.000	2.000	2.000
2.000	2.000	6.000	6.000
6.000	6.000	2.000	2.000
3.000	2.000	3.000	4.000
2.000	2.000	4.000	4.000
2.000	2.000	3.000	3.500
4.000	4.000	6.000	5.500
2.000	2.000		
1.000	2.000		
3.000	2.000	$\bar{x}$ 3.350	3.475
7.000	7.000	SD 1.347	1.342
6.000	5.000	r .849	
1.000	2.000	n = 20	p $<$ 0.001
2.000	2.000		
6.000	6.000		
4.000	3.000		
2.000	2.000		
6.000	4.000		
6.000	6.000		
3.000	3.000		
$\bar{x}$ 3.939	3.727		
SD 2.358	1.941		
r .938			
n = 33	p $<$ 0.001		

Table 14 demonstrating the statistical analyses between the automated and manual techniques.

A total of 202 specimens were compared and the results demonstrate the comparison between the batches. Each batch was tested using completely new reagents including the antigenic solution.

Speed of Sampling (Specimens per minute)	Wash	Number of Specimens	Linear coefficient of correlation
50	1/1	20	0.92
60	1/1	30	0.96
60	1/2	33	0.94
70	1/2	27	0.95
70	1/2	20	0.85
80	1/1	15	0.92
90	1/1	37	0.95
100	1/1	20	0.89

Comparison of all individual pairs of results

$n = 202$ $r = 0.94$ $p = < 0.001$

When a sample was tested 50 times using a speed of 60 per hour the mean ($\bar{x}$) of the specimen was 44.5 increase in percentage transmission (%T), and the standard deviation (SD) 0.9% T. The reproducibility of the system using sera with different titres is illustrated in Fig. 38.

The experiment performed in order to find the influence of the colour on the peak height showed that when sera were tested against unsensitised cells the mean increase was 8%T and SD 1.4%T.

It was found that cells could be sensitised with a solution of milk protein ranging from 200 to 800 mg/100 ml, similarly, cell concentrations ranging from 1.8 to 3.0% gave identical results. Cell concentrations greater than 3% were not practicable due to the raised baseline on the recorder. Satisfactory results were obtained when the speed of sampling was varied from 50 specimens per hour to 100 specimens per hour but the speed used in routine work was 60 specimens per hour.

5.4 Discussion

The automated procedure developed showed correlation with the established manual technique. The influence of the colour of the sera on the total peak height was investigated and found to be consistently less than a titre of $^{1}/_{10}$ and was therefore considered to be of no practical significance. The operator can decide on the speed of testing as a range of sampling speeds and wash-ratios showed correlation. A cell concentration of 3% was used routinely and the cells were coated with a solution of whole dried milk with a protein value of 500 mg/100 ml.

It was considered that this automated method was suitable for practical use. It was realised that with a continual scale of reading, results could be provided which would be superior to the staged levels obtained by the manual double dilution test. This is illustrated and explained in Fig. 39 and new units were

Fig. 38 demonstrating the reproducibility of the automated coated tanned red cell haemagglutination technique. The maximum peak height represents a change of optical density of approximately 70 units.

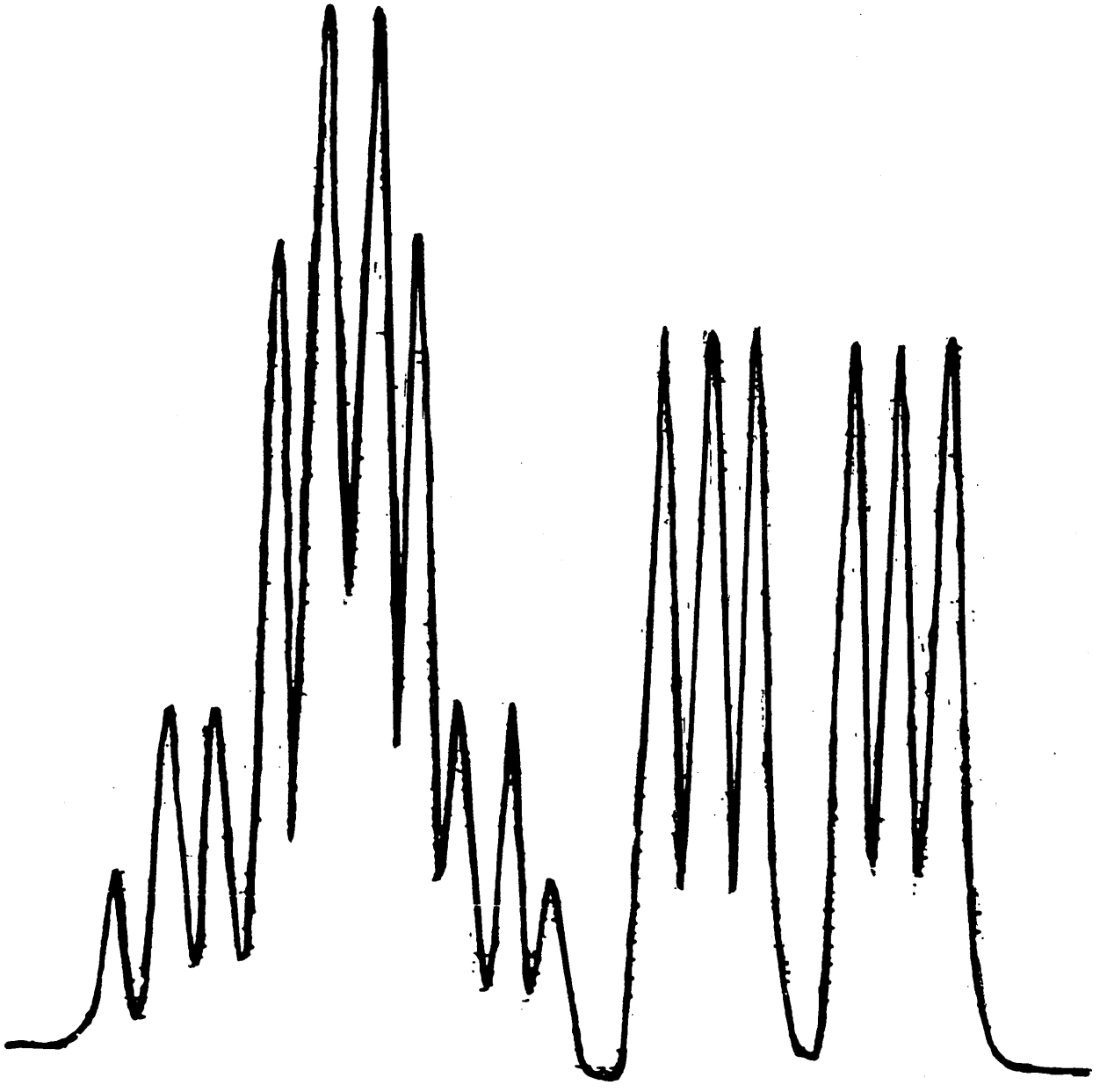
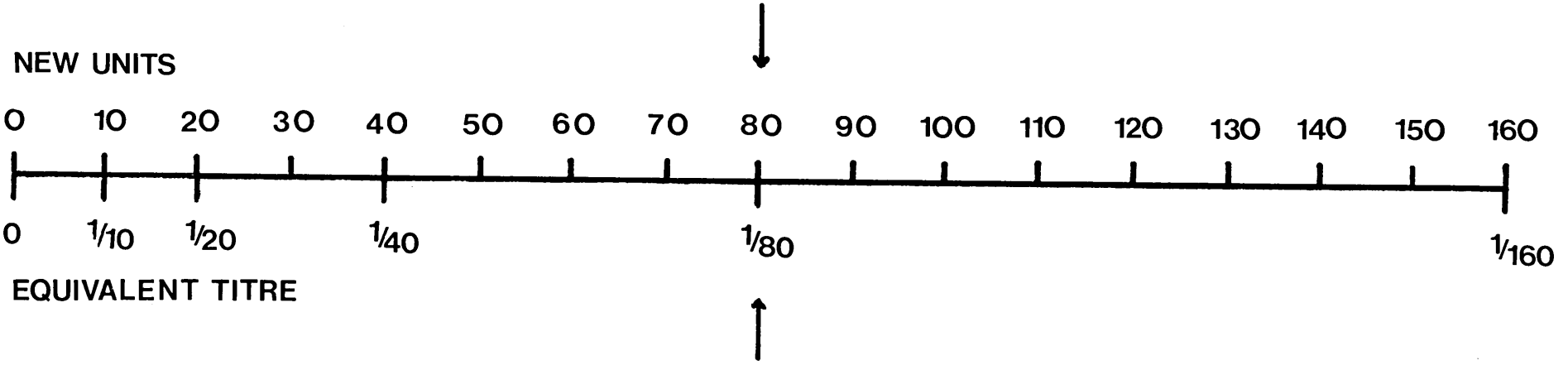


Fig. 39 demonstrating the limitations of the double-dilution non-continuous scale. Using the manual dilution method a positive agglutination at a dilution of $^{1}/_{80}$ and negative at $^{1}/_{160}$ suggests that the result is $^{1}/_{80}$. However, the result could be $^{1}/_{120}$ or $^{1}/_{140}$, without further time consuming dilutions it is impossible to give an accurate titre.

When the titres are converted to the reciprocal of the titres, the "units" give a continuous and more sensitive scale of reading the results. The standards are also converted to these units and therefore the standard graph drawn uses the full potential of the system.


NEW UNITS
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
0 1/10 1/20 1/40 1/80 1/160
EQUIVALENT TITRE

based upon converting the titre into the reciprocal of the titre. This method of reporting the results used the full potential of the instrument and the limitations of the non-continuous scale doubling dilutions were overcome. Subsequently the "standard graph" was drawn from peak heights representing 80, 40, 20 and 10 units and the results of the test sera could therefore be read with greater accuracy.

It was concluded that the automated method could replace the manual haemagglutination test and it was used in the collaborative survey with the MRC. This is described in Chapter 7, page 80.

CHAPTER 6

A STUDY OF AGGLUTINATING ANTIBODIES TO FOOD IN THE NORMAL POPULATION

6.1 Introduction

This chapter describes the immunological response in normal individuals to some commonly eaten foods. A large number of sera from healthy males were available from a population survey performed in conjunction with the MRC and the automated procedure enabled these specimens to be efficiently and accurately titrated. The normal ranges obtained could be referred to and compared with the data obtained in subsequent "case-control" studies.

It has been demonstrated by Schloss (232), Sussman et. al. (233), Lippard (334) and others (235-237) that intact dietary protein derivatives can cross the mature mammalian intestine in sufficient amounts to result in an immunological response and the subsequent production of specific antibodies. This antibody response to various foods, sometimes to a high titre, has been demonstrated in the sera of patients by many workers (172-177).

Proteins are broken down in the intestines to facilitate their absorption and subsequent utilisation by the body. The mechanism by which proteins or protein derivatives are absorbed to stimulate an immune response is reviewed in detail by Muir (183). It would appear that in normal conditions protein derivatives reach the small intestine epithelium as oligopeptides containing 2-3, or probably more, amino acids (238). Kabat (239) has shown that the optimal size of antigenic determinants is 3-6 amino acids which is consistent with these oligopeptides being antigenic. Warshaw and Walker (236) have shown that dietary protein derivatives can be absorbed in antigenic quantities. This occurs in the mature mammalian intestine (237, 240) and it appears that 2% remain antigenically intact (241). The absorption of antigens by the small

intestine is inhibited by antibodies present either on the intestinal surface or possibly within the intestinal lumen (242-243). These antibodies act as receptors to link the antigen to the cell surface proteases, thus immobilising the oligopeptides and aiding their breakdown into amino acids. They are IgA in nature and are considered to be produced by lymphoblasts populating the lamina propria of the intestine. These lymphoblasts are derived from the gut associated lymphoid tissues and are probably precommitted to IgA production before being distributed along the mucosal surface. It has been proposed that specialised epithelial cells, called M cells, provide one route by which antigens from the lumen of the gut are sampled and transported to the lymphoid tissue. After stimulation by the antigen the lymphoblasts are committed to IgA synthesis and they ultimately return to the epithelial surface. The review by Muir (183) also suggests that a partial deficiency in the intestinal antibodies may result in increased absorption of intact antigenic oligopeptides into the blood stream. This could cause the formation of antigen/antibody complexes which would ultimately result in localised inflammation and therefore increased permeability of the gut. This would result in further uptake of antigens reaching the serosal side of the intestine and eventually causing an immune response with the appearance of the specific antibody in the circulation.

The appearance of antibodies in the circulation is related to the method of feeding newborn babies. A newborn infant is deficient in IgA but when it receives colostrum from its mother, it receives antibodies against antigens in the maternal diet, small amounts of antigen and immunocompetent cells. It is therefore immunised both actively and passively to prepare it for its eventual introduction to antigenic food. If, however, the infant is bottle-fed, the antibodies in the cow's milk are destroyed by the heating and drying process and the antigens are given to the infant unopposed. It is known that increased permeability occurs at this time and the antigens can reach the circulation and stimulate antibody response. In this case, re-exposure to minute quantities later in life will result in

a marked increase of the antibody titre. Clearly the presence of antibodies to food in the sera of the adult population warrants further investigation. Lowell (244) states that "there is perhaps no field in medicine in which more divergent views are held than that of response to foods. It would seem worthwhile, therefore, to outline some procedure by which better understanding of the subject be obtained".

This chapter describes experiments performed using the automated tanned red cell haemagglutination technique to investigate the level of antibodies to milk, egg white, meat and gluten in 600 normal healthy males and females.

6.2 Materials and Methods

The investigations were performed on sera from three hundred normal males obtained by the Medical Research Council Unit. These subjects were selected at random from the electoral roll and resided in an area of South Wales covering apprximately 5000 sq. miles. A similar survey of the level of serum antibodies in 300 normal adult females up to the age of 65 was made. These were ambulatory females attending the hospital laboratory for routine blood tests.

Cells were prepared, tanned and sensitised as previously described in Chapter 5, page 64. All the tests were performed using the automated system and the results read in the new units. Preliminary work determined the concentrations for each of the different foods. In this process a wide range of antigenic food concentrations were used to sensitise tanned red cells. Specificity of a positive result was demonstrated by absorbing positive sera with the corresponding antigenic food preparation and repeating the titration. Only after specificity was demonstrated by the reduction of the titre to zero was the test considered specific. Every positive titre reported in this chapter was reduced to zero with the corresponding antigen.

The above development work was performed on random sera available in the laboratory and only after the above criteria were fulfilled were the techniques subsequently used for the population survey. The following sections describe the preparation of various antigenic solutions.

6.2.1 Preparation of milk antigen. The preparation of this antigen has been described in detail in Chapter 5, page 65.

6.2.2 Preparation of gluten antigen. After preparatory work 5 gm of "crude gluten" (Sigma, Poole, Dorset) was added to 15 ml of 0.06M phosphate buffered saline (pH 7.2) (PBS). The solution was stirred and shaken for 15 mins. The mixture was centrifuged at 2400Xg for a further 15 mins. and the supernatant removed and used as the soluble protein solution of gluten. Using the sulphosalicylic acid method (described on page 65) the protein concentration was 300mg/100ml. The sensitised cells demonstrated spontaneous agglutination which was overcome by diluting the final concentration of cells in 1:100 solution of normal serum in PBS.

Random sera were titrated against cells sensitised with the solution of gluten. The negatives were discarded and sera exhibiting positive results were divided into five aliquots and the following added to these tubes containing 1 ml of specimen.

a. 50 mg gluten powder

b. 100 mg white bread

c. 100 mg brown bread

d. 100 mg gluten free bread (Walfare Foods, Stockport, Cheshire).

e. 50 mg whole dried milk as a control

The above were left to react with the sera for 30mins. at room temperature, removed by centrifugation and the sera retitrated against the gluten sensitised cells.

Only after this experiment demonstrated specificity were the 600 normal sera tested for the distribution of antibodies to gluten in the normal population.

6.2.3 Egg-white antigenic preparations. Antigenic preparations were prepared from raw and boiled egg white.

The raw egg whites were separated from the whole eggs and diluted 1:5, 1:10, 1:20, 1:50 and 1:100 with the PBS pH 7.2. Preparatory work showed that the antigenic solution of raw egg could be used at a dilution between 1:5 and 1:100 but for the experiments described in this thesis a dilution of 1:10 was used. This dilution of raw egg was prepared in PBS, shaken for 10 mins., centrifuged and the soluble protein solution removed. Using the sulphosalycilic acid method (described on page 65) the protein value of this antigen solution was approximately 300mg/100ml.

The cells were tanned and sensitised with this antigenic solution using the same method as described for the milk antigen.

In addition to the raw egg, solutions of boiled egg antigens were also prepared. One egg was boiled for 10 mins. and the white separated from the yellow. The egg white was chopped into small pieces, placed in a liquidiser and 20 ml of the PBS added. After liquidising for 5 mins. the solution was transferred to a suitable tube and centrifuged at 2400Xg for 15 mins. The supernatant was removed and this soluble proteinof egg white was found to have, on average, a protein concentration of 280mg/100ml. The tanned cells were prepared and sensitised with this antigen as previously described.

6.2.4 Preparation of meat antigens. Protein solutions prepared from raw and cooked beef, lamb and pork were tested in this experiment.

When preparing a solution of raw meat, the surface blood

was recovered by washing in PBS. The meats were liquidised with varying amounts of PBS, the solutions centrifuged and the supernatants used as the solutions of protein. The cells were sensitised with these preparations using the method previously described.

For the cooked meat antigen the meat was roasted in the oven in the usual manner and when cold, a portion was removed and a solution of antigens prepared as described for the raw meat.

Raw and cooked protein solutions were obtained from all the different meats listed and a wide range of protein concentrations prepared from each individual solution. Cells were sensitised with these different concentrations and each batch tested against the 300 normal male and the 300 normal female sera using the automated method.

6.3 Results

6.3.1 The distribution of antibodies to whole dried milk in the normal population. The results from the 300 sera of healthy males can be seen in the histogram illustrated in Fig. 40. The mean titre was 15.4 units and the standard deviation 24.7.

The distribution of antibodies to whole dried milk in the 300 females tested is seen in the histogram showing in Fig. 41. The mean titre was 29.5 units and the standard deviation 42.5.

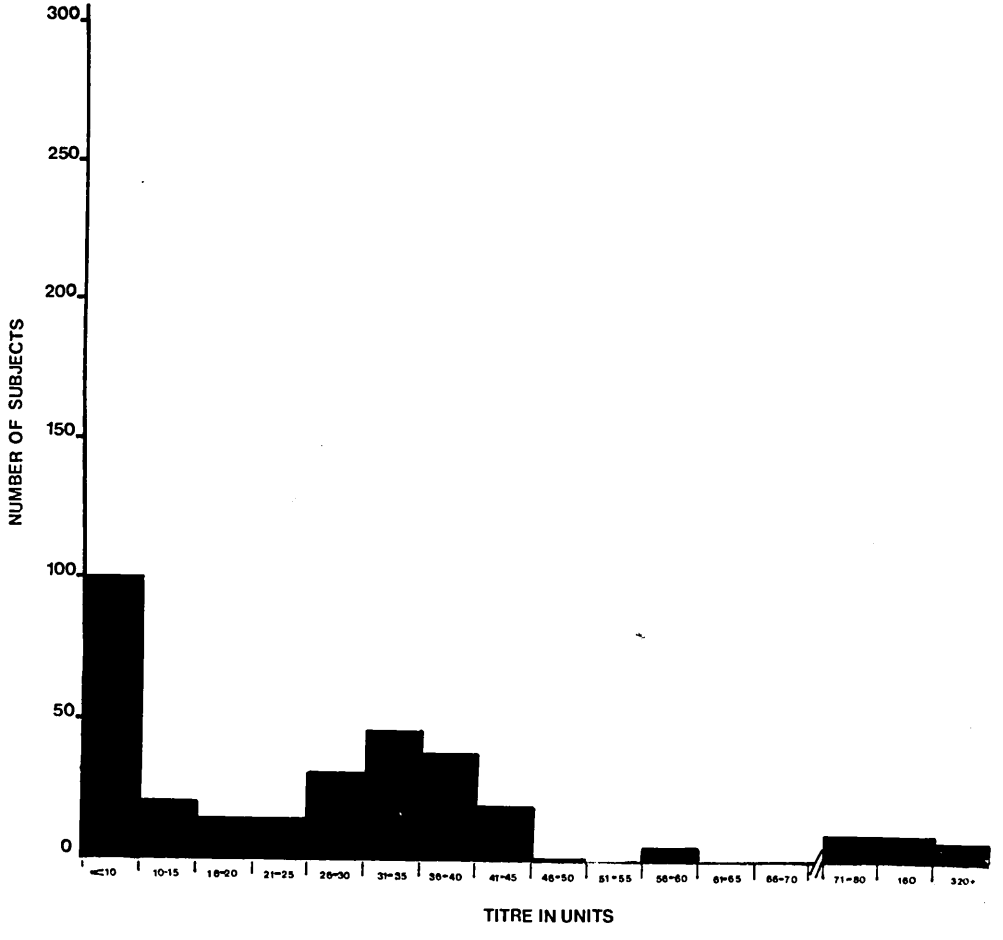
These two figures (40 and 41) illustrate that a higher percentage (67% with a titre greater than 10 units) of females have antibodies to dried milk when compared with normal males (36% with a titre greater than 10 units). The actual antibody level was also higher in the female (mean level was 29.5 units compared with 15.4 units in males). The correlation 'r' between these two groups was 0.50. This compared with values of greater than 0.94 for all the other foods tested.

Fig. 40 showing the distribution of antibodies to whole dried milk in the sera of 300 normal healthy males.

The mean titre = 15.4 units and the standard deviation 24.7 units.

Fig. 41 demonstrating the distribution of antibodies to whole dried milk in the 300 normal female sera tested.

The mean titre = 29.5 units and the standard deviation 42.5.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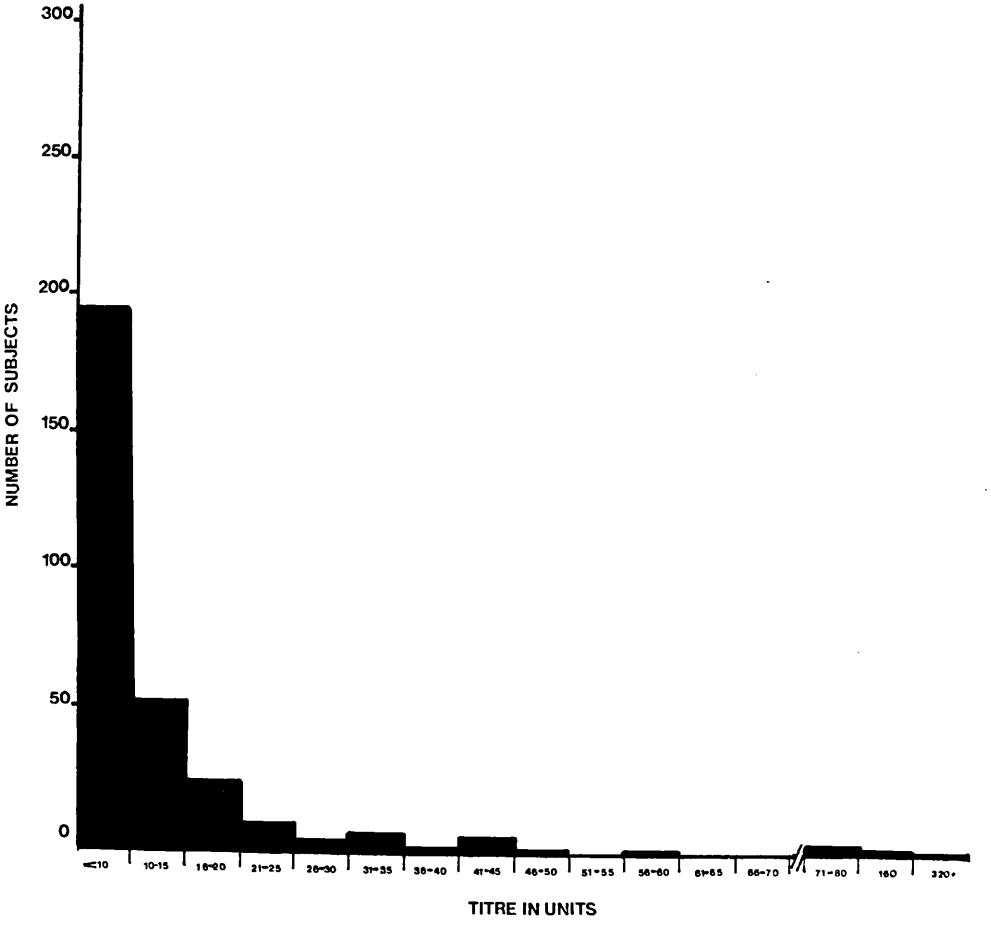
TITRE IN UNITS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320+
TITRE IN UNITS

6.3.2 The distribution of antibodies to gluten in the normal population. The experiment involving the absorption of sera demonstrating a titre to gluten with different preparations of bread showed a reduction in titre in many of the preparations tested. The titres were reduced to less than 10 units with the gluten powder, white bread and brown bread but antibody remained in the sera absorbed with the gluten-free bread and the control tube of dried milk. This demonstrated the specificity of the method. Histograms demonstrating the distribution of antibody to gluten in 300 normal males are illustrated in Fig. 42 and normal females in Fig. 43. The mean titre for the male sera tested was 13.8 units and the standard deviation 20.9. Statistical analyses on the sera from the normal females showed that the mean value was 13.9 units and the standard deviation 22.3. Analyses to compare the two groups showed a correlation (r) value of 0.95 which was highly significant ($p < 0.001$).

6.3.3 The distribution of antibodies to egg white in the normal population. The distribution of antibodies to boiled egg white in 300 normal males can be seen in the histogram illustrated in Fig. 44 and the normal females in Fig. 45. The mean titre for the male sera was 10.0 units and the standard deviation 3.6. A mean value of 10.0 was also obtained for the female sera tested and the standard deviation was 3.5. A correlation of 0.97, which was highly significant ($p \leq 0.001$), was obtained when the two groups were compared.

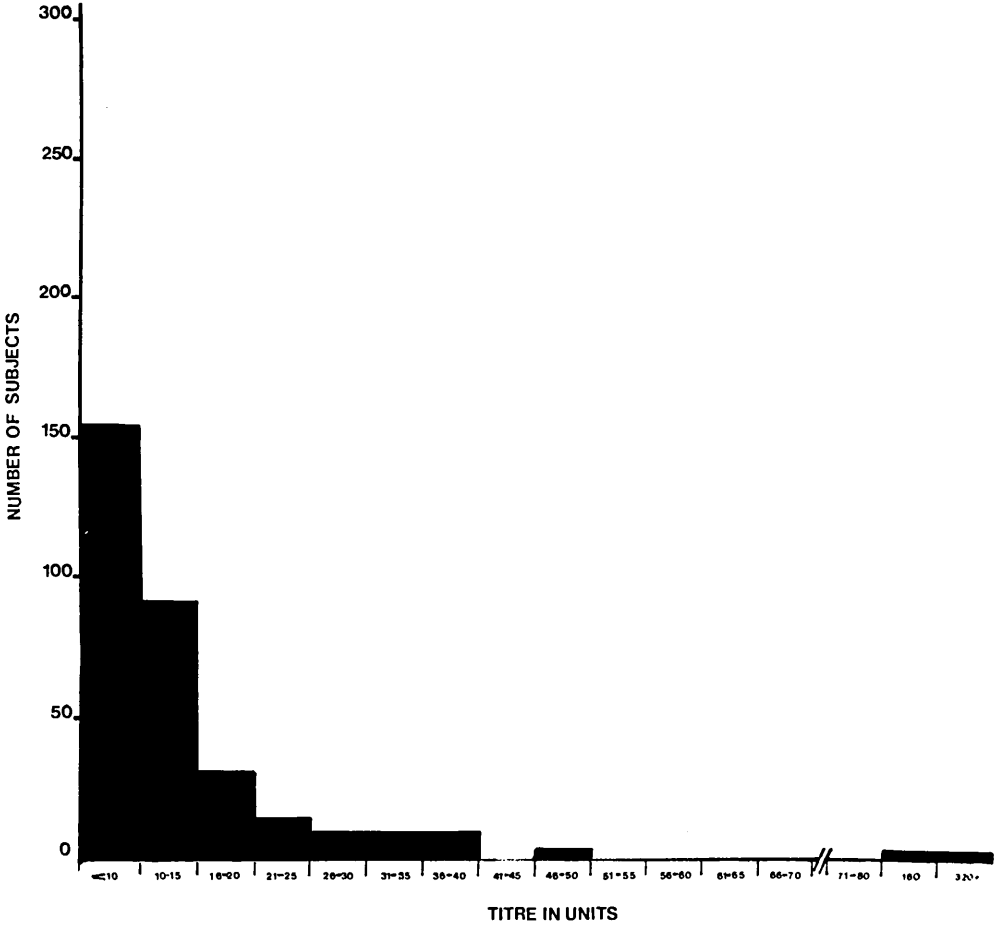
The antibody distribution to raw egg white in the normal males can be seen in Fig. 46. The mean titre was 9.2 units and the standard deviation 2.9. The histogram showing the distribution of antibodies to raw egg white in the females is illustrated in Fig. 47. The mean value in this group was 9.1 units and the standard deviation 2.8. A correlation (r) of 0.94 was obtained when the two groups were compared. This was highly significant ($p < 0.001$).

Fig. 42 demonstrating the distribution of antibodies to gluten in 300 normal males.

The mean value was 13.8 units and the standard deviation 20.9.

Fig. 43 demonstrating the distribution of antibodies to gluten in 300 normal females.

The mean value was 13.9 units and the standard deviation 22.3.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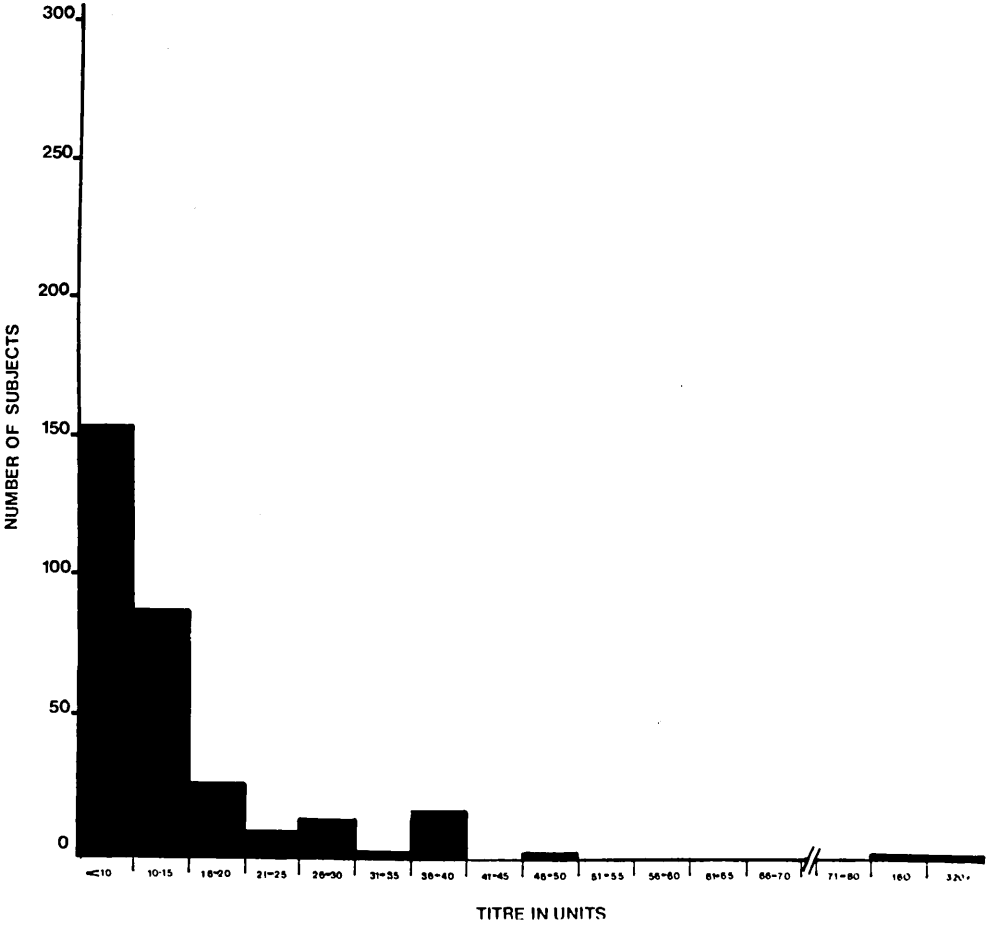
TITRE IN UNITS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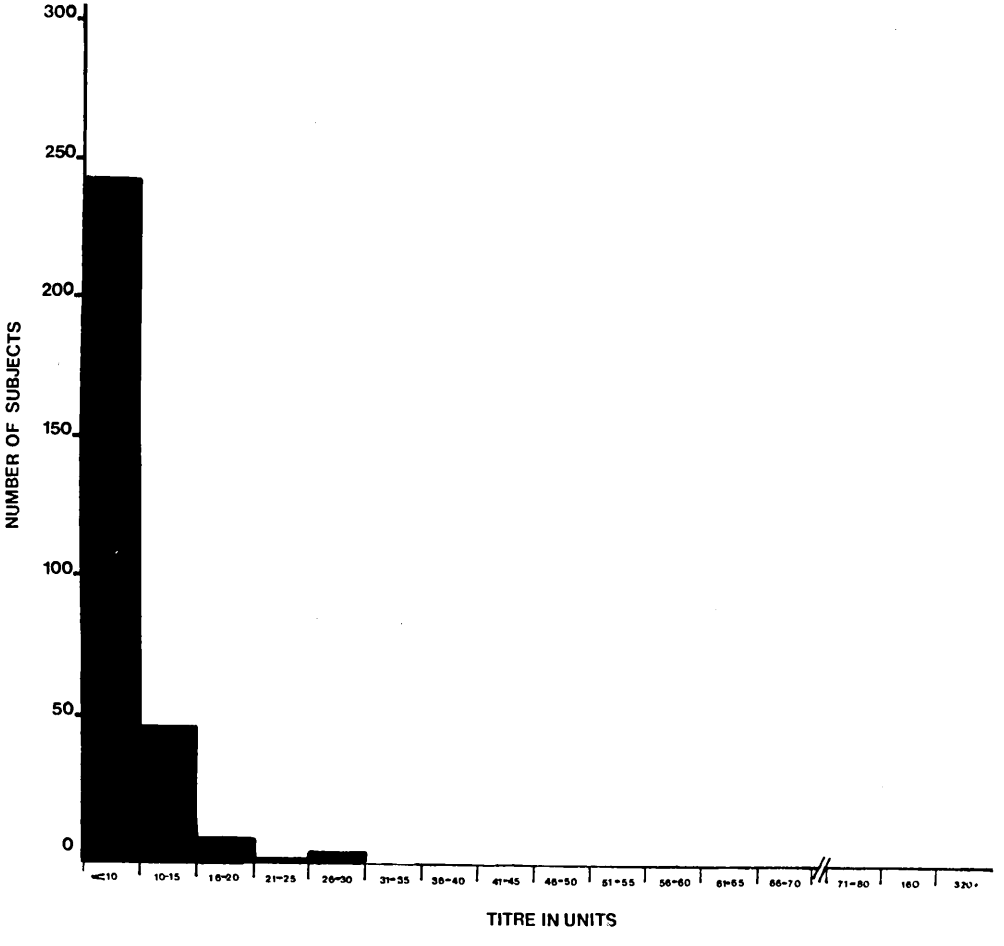
160
TITRE IN UNITS

Fig. 44 demonstrating the distribution of antibodies to boiled egg white in 300 normal males.

The mean value was 10.0 units and the standard deviation 3.6.

Fig. 45 demonstrating the distribution of antibodies to boiled egg white in 300 normal females.

The mean value was 10.0 units and the standard deviation 3.5.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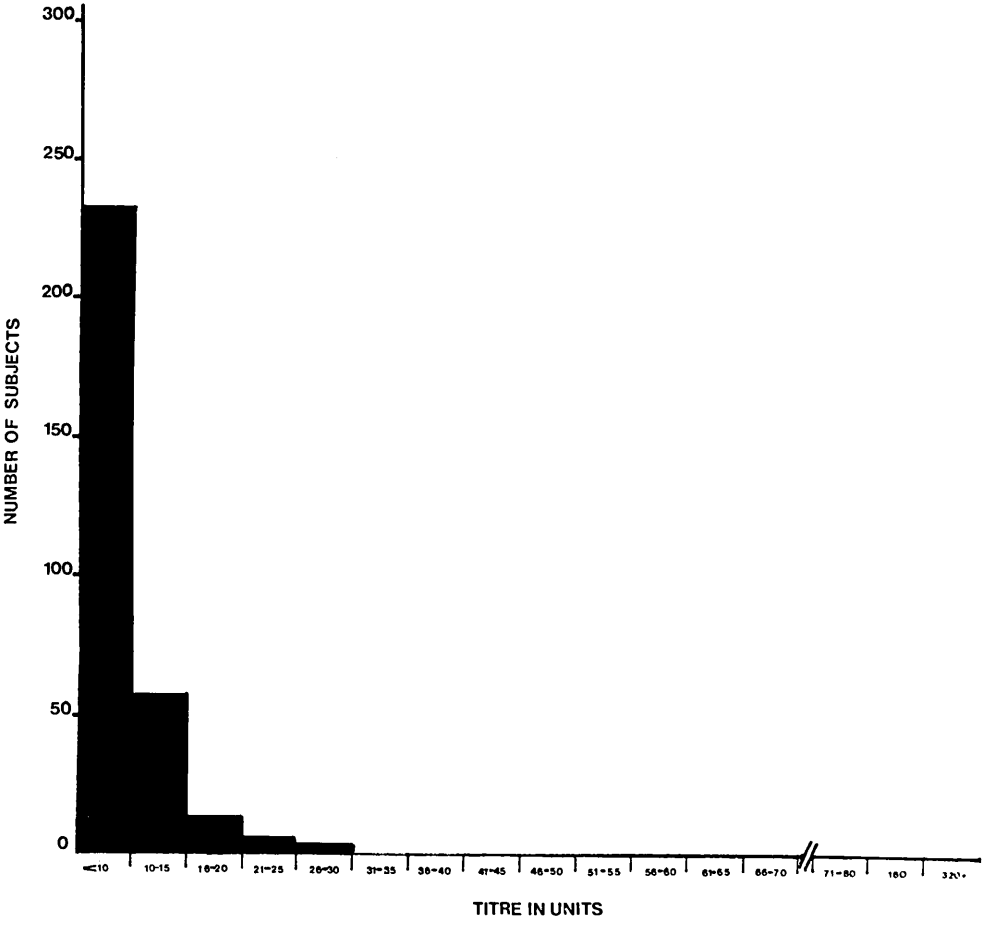
TITRE IN UNITS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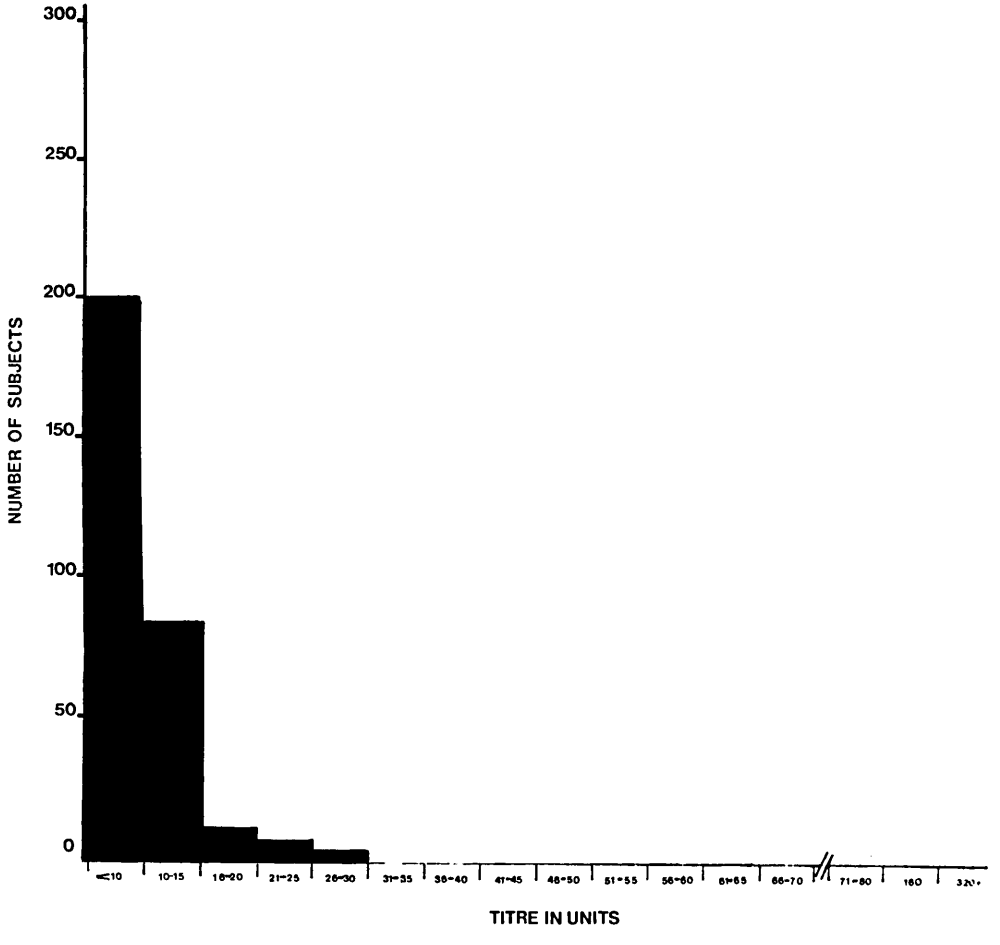
TITRE IN UNITS

Fig. 46 demonstrating the distribution of antibodies to raw egg white in 300 normal males.

The mean value was 9.2 units and the standard deviation 2.9.

Fig. 47 demonstrating the distribution of antibodies to raw egg white in 300 normal females.

The mean value was 9.1 units and the standard deviation 2.8.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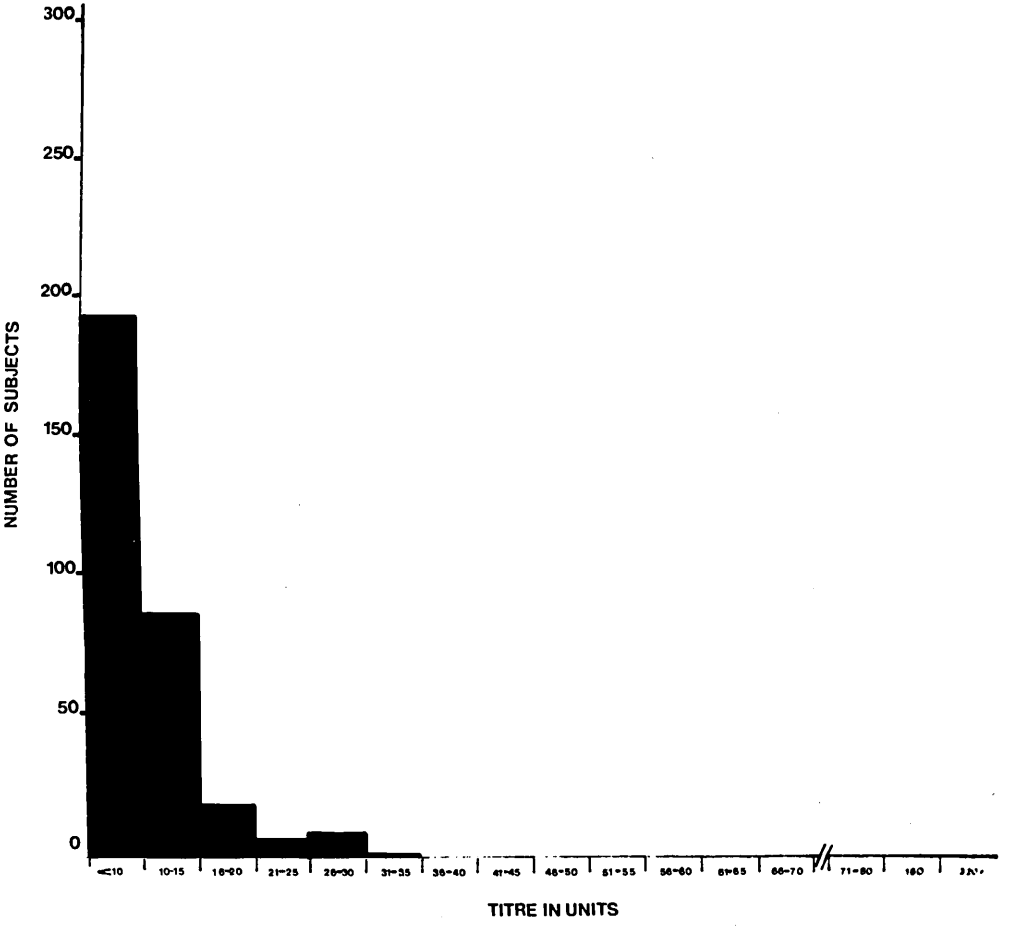
TITRE IN UNITS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TITRE IN UNITS

6.3.4 The distribution of antibodies to meat in the normal population. No antibody response was obtained in the 300 normal males and the 300 normal females to antigenic preparations from raw and cooked beef, pork or lamb.

The summary of the results obtained in this section is illustrated in Table 15.

6.4 Discussion

This chapter describes the antibody response in the normal population to various foods. The foods chosen for testing included some of the most commonly consumed. They were milk, gluten, egg and meat. The subjects used to determine the distribution consisted of healthy males chosen by the MRC from an extensive area of South Wales and 300 normal females attending the laboratory out-patients' department.

The results obtained when testing the antibody level in response to whole dried milk demonstrated that the female population appears to show a considerably higher titre than those observed in the normal males tested. Many factors could explain this difference. Preliminary work showed that one factor was pregnancy and further experiments are warranted on this aspect. However, the investigation of the sex related difference is beyond the scope of this thesis. It does, however, underline how essential it is to confine, at this stage, related work to males only.

Despite the differences now demonstrated, females of child bearing age have a lower incidence of coronary heart disease than males (245). A review in the "Lancet" (245) suggested that one possible explanation was that the increased endogenous oestrogen production from an early age might prevent the development of the disease. However, the incidence of CHD disease in females increases after the menopause (246, 247). While some protective mechanism from CHD may exist in the younger female, the incidence of venous thrombosis in this group

Table 15 summarising the correlation between the distribution of antibodies to milk, egg and gluten in 300 males and 300 females. The titrations were performed using the automated tanned red cell haemagglutination technique.

	Antibody titrated	Males	Females
	Whole Dried Milk		
Mean S.D. r		15.4 24.7 0.56	29.5 42.5
	Gluten		
Mean S.D. r		13.8 20.9 0.95	13.9 22.3
	Boiled egg white		
Mean S.D. r		10.0 3.6 0.97	10.0 3.5
	Raw egg white		
Mean S.D. r.		9.2 2.9 0.94	9.1 2.8
	No antibodies were detected against the meat antigens tested.		

is far higher than in men from the same age group (248).

The experiment involving gluten showed that some of the normal population produce antibodies to this antigen. No significant difference was encountered between the response in the 300 male and 300 female sera tested.

The response to raw and cooked egg white in the normal population demonstrated that some subjects produce an antibody to these antigens. No difference was encountered between the response of the male and female population to raw egg or boiled egg white. However, there was no obvious relationship between the raw and boiled egg white titres in individual subjects. Further experiments demonstrated that the raw egg antigen did not absorb the boiled egg antibody and the reverse of this was also true. This suggested that these two antibodies are produced by different antigens, one to raw egg white and the other to boiled egg white.

No antibody response to raw and cooked meat antigens was found. But it may remain possible that the procedure used for antibody determination is not suitable for the demonstration of antibodies to meat.

The results described in this chapter demonstrate that a percentage of the normal population produce antibodies to food antigens. All the sera were tested with the automated tanned red cell haemagglutination method, developed and described in the previous chapter.

CHAPTER 7

FOOD ANTIBODIES AND CORONARY HEART DISEASE

7.1 Introduction

This chapter describes a survey performed primarily to test the observation that coronary heart disease is related to the development of antibodies to whole dried milk when compared with controls matched for age (178). This survey was performed with the co-operation of the MRC Unit. A large number of confirmed MI patients and control subjects were investgated from a wide area (approximately 5,000 square miles) of South Wales. Use was made of the automated procedure for titrating the sera. In addition to whole dried milk, gluten and egg white were investigated.

A small sub-sample of paired sera was investigated for antibodies to various preparations of soya bean. This was the result of the observations of Taylor (249) who used rabbits to investigate the immunological theory of coronary heart disease. The report by Muir et. al. (249) stated "preliminary experiments are consistent with the view that the formation of antibodies to dietary proteins may indeed constitute a risk factor in the development of atheroma in rabbits".

7.2 Materials and Methods

All the sera tested were received from the staff of the Medical Research Council Epidemiology Unit, Cardiff. A total of 216 patients who had suffered a myocardial infarction and 144 control subjects were tested. The sera were tested "blind" with regard to diagnoses and the results were returned to the MRC for statistical analyses. Strict diagnostic criteria, detailed in Chapter 2, page 30, were maintained by one medical consultant physician under the direction of the MRC. Technical proecedures have been described in detail in Chapter 6 page 73.

7.2.1 Preparation of soya bean antigen. The soya bean preparations tested were obtained from Professor Taylor (Department of Science and Nutrition, University of Southampton). Five preparations were tested; proteinate, soyolk, soygold, promine D and bronsoy 100.

Preliminary work showed that suitable solutions could be prepared by dissolving 3 grm of the soya bean preparations in 20 ml 0.06M phosphate buffered saline (pH 7.4) (PBS). This was shaken for 10 mins. and centrifuged at 2400Xg. The tanned cells were sensitised with these preparations of protein and every positive result obtained was absorbed with the specific antigen. The subsequent titration showed the reduction of the titre to less than 10 units, confirming specificity.

7.3 Results

The antibody titres to whole dried milk, gluten, egg white and soya bean were assayed "blind" and the results sent to the MRC Unit for statistical analyses. The data was processed and subsequently published in the Lancet (200). In brief, the conclusions were:-

1. It was confirmed that a higher percentage of men who had suffered a myocardial infarction possessed antibody to whole dried milk when compared with control subjects.

2. Mortality within six months after infarction in the group of patients possessing milk antibody was 28% compared with 10% in patients with no antibodies.

3. Antibodies to egg white also appeared to have a relationship with CHD. This observation is discussed further in the "General Discussion" chapter of this thesis.

4. No difference between the two groups was observed in

antibodies to gluten.

5. No detailed analyses were received from the MRC on the small sub-group tested with the five different preparations of soya bean. However it was stated by the MRC statisticians that there were no significant findings. It is considered that no firm conclusions can be made from this investigation and a further, larger study is indicated.

This chapter describes the confirmation of the original observations of Davies and his co-workers (178), showing that a relationship exists between the presence of antibody to whole dried milk and coronary heart disease. It was also concluded that the presence of this antibody was related to mortality in such patients.

CHAPTER 8

INVESTIGATIONS CONCERNING ANTIBODY TO "WHOLE DRIED MILK"

8.1 Introduction

The previous chapter describes investigations performed in conjunction with the Medical Research Council Epidemiology Unit which confirmed the earlier work of Davies et. al. (178) in which it was shown that antibody to whole dried milk was related to coronary heart disease. Prior to that stage it had been considered essential to study the original antigenic preparation used by Davies (178) although it was known that it contained a mixture of many milk proteins. This chapter describes investigations performed attempting to identify and isolate the individual protein(s) responsible.

The investigations commenced by testing patients' sera against cells sensitised with the three main protein fractions of milk; casein, α-lactalbumin and β-lactoglobulin. The possibility of the antigen involved being the result of the denaturation of milk proteins during the drying process was tested by comparing the antibody titres obtained with cells sensitised with fresh whole milk. The effect of the cream portion of milk was investigated by centrifuging whole fresh milk and removing the cream. The tanned cells were sensitised with the fresh milk with the cream removed.

These investigations suggested that the antigen(s) responsible for the production of the antibody, previously loosely described as "milk antibody", was located in the cream portion of milk. It is the aim of this chapter to isolate and identify the fraction of cream implicated in coronary heart disease. Because of the nature of milk-fat globule-membrane (250), it was thought possible that this could be the antigen responsible for the production of the "milk antibody". These investigations and their significance are detailed in this chapter.

8.2 Materials and Methods

8.2.1 Comparison between the antibody levels to dried whole milk and the three main protein components. Solutions of casein, α-lactalbumin and β-lactoglobulin (kindly supplied by Dr. Cheeseman, Institute for Research in Dairying, Reading University) and whole dried milk were prepared in 0.06 phosphate buffered saline (pH 7.2) (PBS) and the protein concentrations adjusted to 500 mg/100 ml. Cells were tanned and sensitised with solutions of these proteins. A total of 100 sera were titrated using the automated system and the results compared.

Absorption experiments were also performed. Sera exhibiting a titre to dried whole milk were divided into 5 aliquots of 0.5 ml. A volume of 0.1 ml of the antigenic preparations from the dried whole milk, casein, α-lactalbumin and β-lactoglobulin were added to each of four tubes. The same amount of saline was added to one further aliquot, as a control. The sera were mixed, left at room temperature for 30 mins. and tested against cells tanned and sensitised with whole dried milk using the automated system already outlined.

8.2.2 Comparison between the antibody levels to whole dried milk and whole fresh milk. Whole fresh milk was obtained from a local farm. A portion of this milk was centrifuged at 2400Xg for 20 mins. and the cream portion removed. Cells were tanned and sensitised with (a) a solution of reconstituted whole dried milk; (b) whole fresh milk; and (c) freshly prepared skimmed milk. A total of 120 sera were tested. In addition, absorption experiments were performed. A suspension of cream was added to the sera exhibiting a titre to both the whole dried milk and the fresh whole milk antigens. After 30 mins. at room temperature the titrations were repeated against cells sensitised with the whole dried milk and the whole fresh milk.

8.2.3 Comparison between the antibody levels to whole dried milk, washed fresh cream and commercially available skimmed milk. Fresh milk was obtained from a local farm, allowed to settle and the supernatant cream removed. This was washed three times in PBS and cells were tanned and sensitised with this antigenic suspension. A solution of commercially prepared skimmed milk was prepared by dissolving 3 grm of the powder in 20 ml PBS. This was centrifuged at 2400Xg for 20 mins. and the clear solution removed and used to sensitise the tanned red cells.

All subsequent tanned red cell haemagglutination tests described in this chapter were performed using a commercially available automated method - the Dynateck Rotator System (Dynateck Laboratories Ltd., Danx Road, Billingshurst, Sussex). Sera were double diluted from neat and the sensitised cells added automatically. Micro-haemagglutination trays were used and the results read and recorded as previously described in detail for the manual method (page 63).

8.2.4 Comparison between the antibody levels to whole dried milk and milk-fat globule-membrane. The method of preparing the bovine milk-fat globule-membrane was based on that described by Newman (251). Briefly, fresh cream was washed with large volumes of PBS and this washed cream placed at 40°C. After 30 mins. the butter oil was removed and the milk-fat globule-membrane used to sensitise tanned cells.

8.2.5 Comparison between the tanned red cell haemagglutination technique and another method of antibody detection. All the observations made and subsequently published were based on results obtained with the sensitised tanned red cell haemagglutination technique. This section describes experiments designed to validate these findings using the enzyme-immuno assay (EIA)* method of antibody detection. This was performed in collaboration with Flow Laboratories (Flow Laboratories, Second Avenue Industrial Estate, Irvine). The principle of

*subsequently retitled ELISA or enzyme-linked immunosorbent assay.

the EIA method depends on the fact that antigen can be absorbed and bound to an inert carrier surface, such as the wells of micro-titration plates, without losing activity. Subsequently, after washing, the test sera are added to these trays. The specific antibody attaches to the 'prepared' antigenic surface. After washing, enzyme labelled antiglobulin of the relevant species is added to these trays. The specific antiglobulin antibody attaches itself to the antiglobulin and after further washing a substrate is added which changes colour in proportion to the enzyme remaining. Therefore the amount of antibody present is directly proportional to the optical density of the final colour.

Sera were titrated in the author's laboratory using tanned cells sensitised with solutions of whole dried milk and also milk-fat globule-membrane. An aliquot of the same sera and the antigenic solutions were sent to Flow laboratories and the antibody levels were assayed there using the EIA method.

8.3 Results

8.3.1 Comparison between whole dried milk and the three main milk protein fractions. Examples of the results obtained are illustrated in Table 16. The total number of sera tested was 100 and the correlation between the titres of dried whole milk and casein was r 0.22 p = 0.9, dried milk and α-lactalbumin r 0.22 p = 0.9 and dried milk and β-lactoglobulin r=0.22 p = 0.9.

Absorption experiments showed that of the 20 sera exhibiting a titre to dried whole milk; an antigenic solution of dried whole milk reduced the level to less than 10 units in all the specimens tested. No reduction of the titre of the antibody to whole dried milk was observed after solutions of casein, α-lactalbumin or β-lactoglobulin were added to the sera;

Table 16 showing examples of the results obtained when the same sera were tested against whole dried milk and the three main proteins of milk:- casein, β-lactoglobulin and α-lactalbumin.

A lack of correlation ($r = 0.22$, $n = 100$) was observed between the whole dried milk and the three fractions. This was reflected in a p value of 0.9 ($t = 2.2$)

Serum Number	Whole Dried Milk	Casein	β - Lactoglobulin	α - Lactalbumin
1	11	8	8	8
2	8	9	12	9
3	20	12	11	12
4	11	8	8	8
5	9	8	8	9
6	9	8	7	7
7	15	8	6	6
8	100	10	9	9
9	100	9	10	10
10	120	15	11	8
11	15	8	8	5
12	10	9	6	8
13	10	8	7	8
14	6	8	8	8
15	10	8	8	8
16	8	8	8	8
17	35	10	8	6
18	15	10	8	6
19	20	8	18	7
20	10	8	16	12
21	10	8	10	8
22	10	9	10	8

these results were the same as those obtained when the sera were 'absorbed' with saline.

8.3.2 Comparison between dried whole milk and fresh whole milk. Examples of the comparison between the whole dried milk and the whole fresh milk titres can be seen in Table 17. A total of 120 sera were tested and $r = 0.99$ $p = < 0.001$. Cross absorption experiments confirmed that whole dried milk absorbed to less than 10 units the antibody to whole fresh milk. The antibody to whole dried milk was also absorbed to less than 10 units by whole fresh milk.

Some examples of the results obtained with sera tested against cells sensitised with dried whole milk and fresh whole milk with the cream removed are also illustrated in Table 17. A total of 120 sera were tested and $r = 0.28$ and $p = 0.7$.

Investigations into the effect on the titre of antibodies after the addition of cream showed that antibodies to whole dried milk and whole fresh milk were reduced to less than 10 units after absorption with cream. Examples of these results can be seen in Table 18.

8.3.3 Comparison between the antibody titres to whole dried milk, washed cream and dried skimmed milk. The distribution of the results of 50 sera tested against whole dried milk and washed cream can be seen in Fig. 48. Correlation "r" was 0.96 which is highly significant ($p = < 0.001$). The results obtained with skimmed milk showed $r = 0.50$. This is illustrated in Fig. 49.

Absorption experiments confirmed that washed cream reduces the level of antibody to whole dried milk to zero. The antibody titres obtained with washed cream were also reduced to zero with whole dried milk.

Table 17 showing examples of the correlation between the whole dried milk and the whole fresh milk.

A significant correlation ($r = 0.99$, $n = 120$) was observed between these two preparations. A p value of < 0.001 ($t = 76$) was highly significant.

Also included are the results obtained when tanned cells were sensitised with whole fresh milk but with the cream removed. A lack of correlation ($r = 0.28$, $p = 0.7$, $t = 3.0$) is observed between the results for this antigenic solution and those for whole dried milk.

Specimen No.	Whole dried milk	Fresh whole milk	Fresh whole milk with cream removed
1	26	24	10
2	6	6	6
3	30	28	10
4	40	42	10
5	6	6	6
6	160	152	36
7	7	8	7
8	7	8	7
9	320	340	80
10	16	16	8

Table 18 showing examples of the results obtained when sera exhibiting a titre to whole dried milk were "absorbed" with saline and also absorbed with the same volume of cream.

Every specimen was absorbed to less than 10 units with the cream when the sera were tested with cells sensitised with an antigenic solution of whole dried milk.

Specimen No.	Sera "absorbed" with saline	Sera absorbed with cream
1	26	9
2	46	6
3	30	6
4	40	6
5	16	8
6	160	9
7	27	7
8	17	7
9	320	9
10	16	8

Fig. 48 showing the correlation obtained when testing sera for antibodies to whole dried milk and washed cream.

A correlation (r) of 0.96 was obtained and this was highly significant ($p < 0.001$).

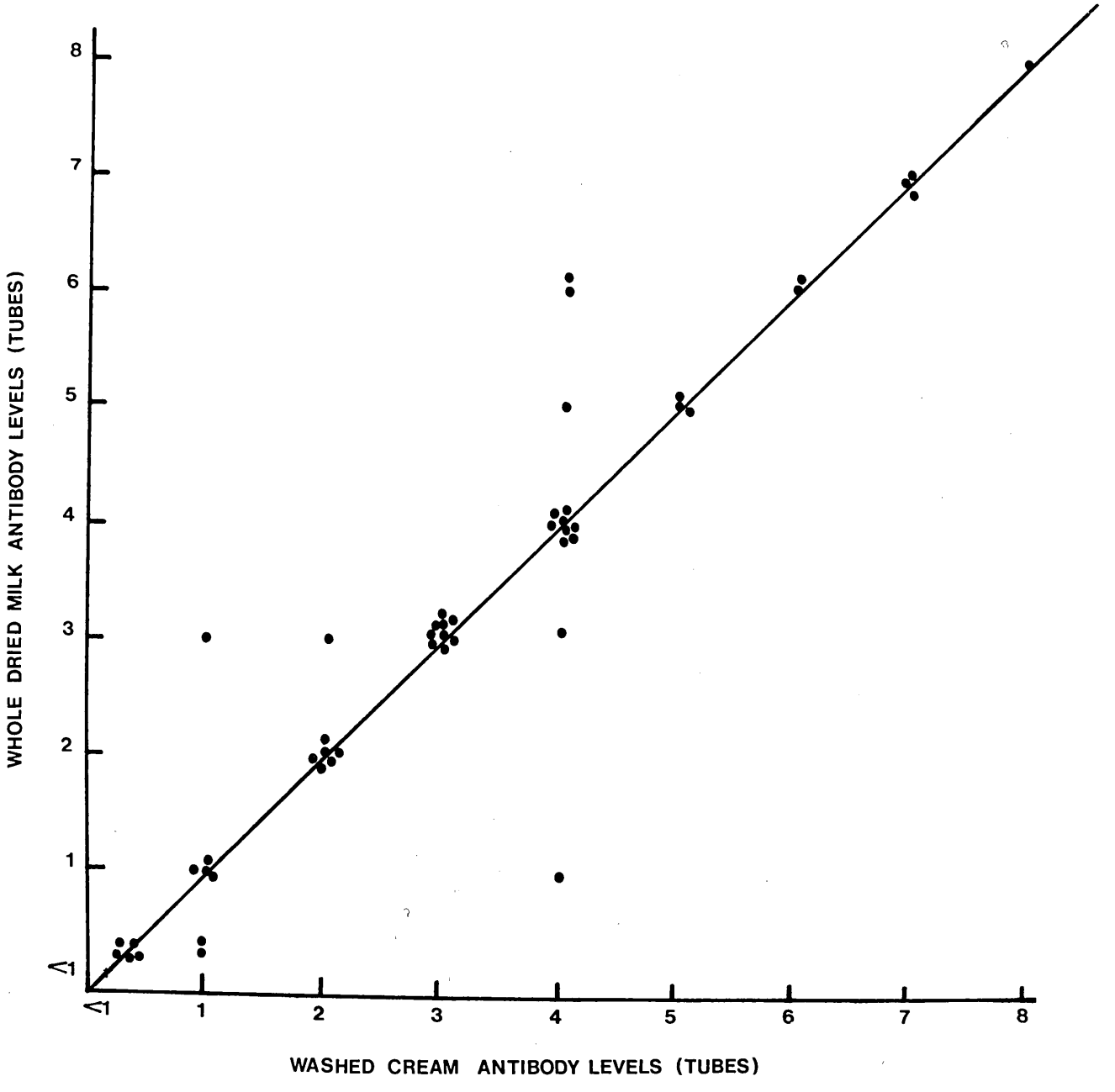
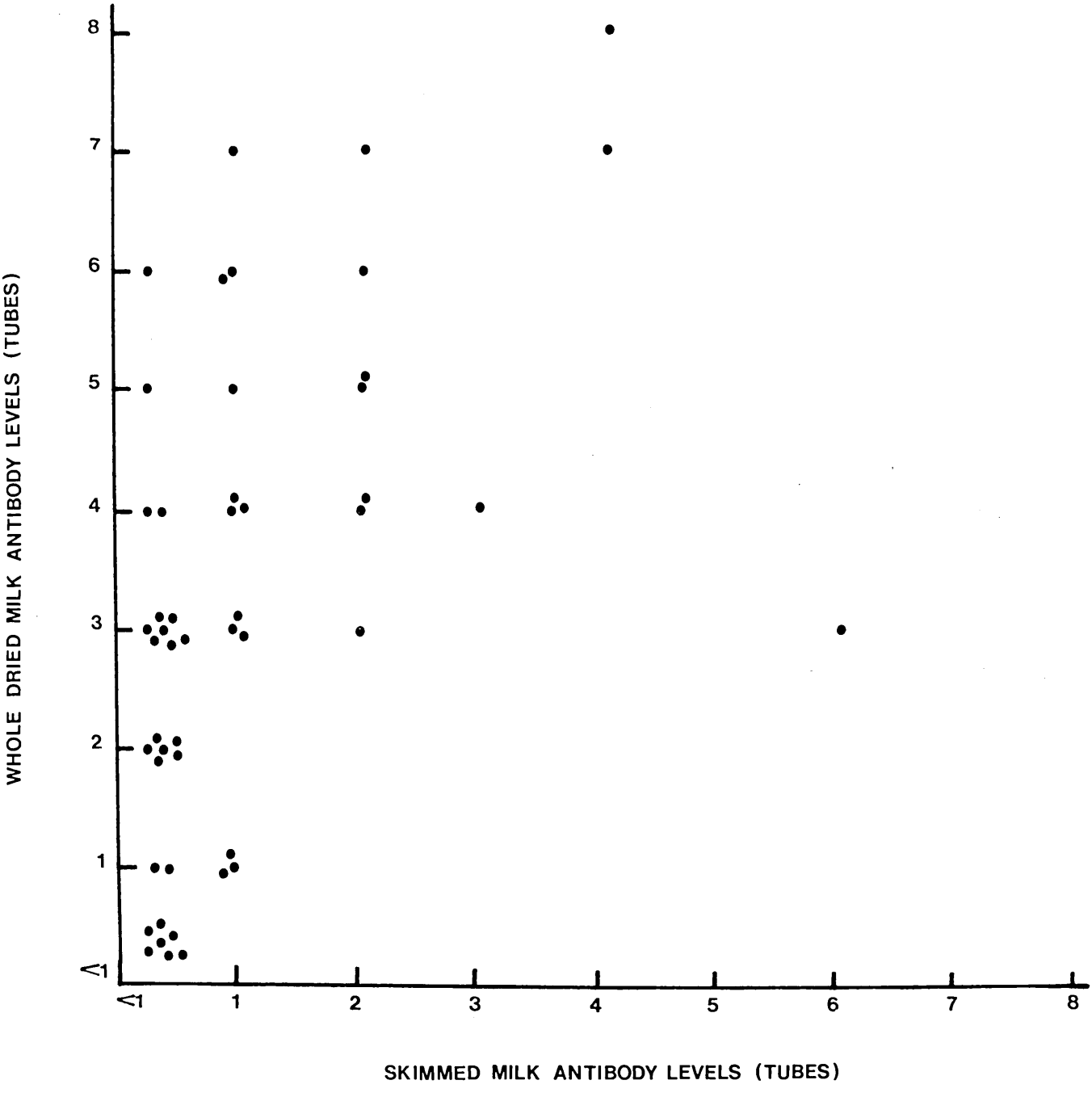

WHOLE DRIED MILK ANTIBODY LEVELS (TUBES)
WASHED CREAM ANTIBODY LEVELS (TUBES)
<1
1
2
3
4
5
6
7
8

Fig. 49 showing the scatter diagram obtained when the same sera were titrated for antibodies to whole dried milk and skimmed milk.


WHOLE DRIED MILK ANTIBODY LEVELS (TUBES)
8
7
6
5
4
3
2
1
<1
<1
1
2
3
4
5
6
7
8
SKIMMED MILK ANTIBODY LEVELS (TUBES)

8.3.4 Comparison between the antibody titres to whole dried milk and milk-fat globule-membrane. Titration of 200 sera was performed and sera showing a negative result to both antigens have been excluded from the statistical analyses. The correlation between the remaining 48 paired results is demonstrated in Fig. 50 and $r = 0.95$ $p = < 0.001$.

Cross-absorption experiments showed that the bovine milk-fat globule-membrane antigen reduced the level of the antibody to whole dried milk to zero. The antigenic solution of whole dried milk had the same effect on the antibody titre to the bovine milk-fat globule-membrane.

8.3.5 Comparison between the antibody titres to whole dried milk and milk-fat globule-membrane using the EIA method. The results of the collaborative study with Flow Laboratories are illustrated in Fig. 51. Shown are the levels obtained with the EIA method of sera tested against whole dried milk and bovine milk-fat globule-membrane. Correlation 'r' of 0.97 ($p = \leq 0.001$) was obtained.

8.3.6 Comparison between the haemagglutination technique and the EIA method. Specimens were tested for levels of antibodies against bovine milk-fat globule-membrane, using the tanned red cell haemagglutination technique, in the author's laboratory. The same sera were tested by Flow laboratories using the EIA method. The EIA results were reported in optical density (OD) units. The data from the preliminary experiment is listed in Table 19. It appeared from the data that when the haemagglutination result was $^1/_{16}$ or greater, the EIA test was always higher than 0.10. This was also found in five out of nine of the haemagglutination results of $^1/_8$. However, in the lower dilutions, it was obvious that many sera exhibiting a negative haemagglutination result showed the presence of an antibody with the EIA method. In a lower percentage, the reverse of this was found. In the second collaborative study performed, it was decided to examine the data in terms of positive/negative results. ODs of < 0.100 were accepted as negative, 0.100-0.150 as positive/negative and >0.150 as definite positives. This

Fig. 50 demonstrating the correlation between the results of sera tested against whole dried milk and bovine milk fat globule-membrane. A correlation 'r' of 0.95 was obtained. ($p < 0.001$).

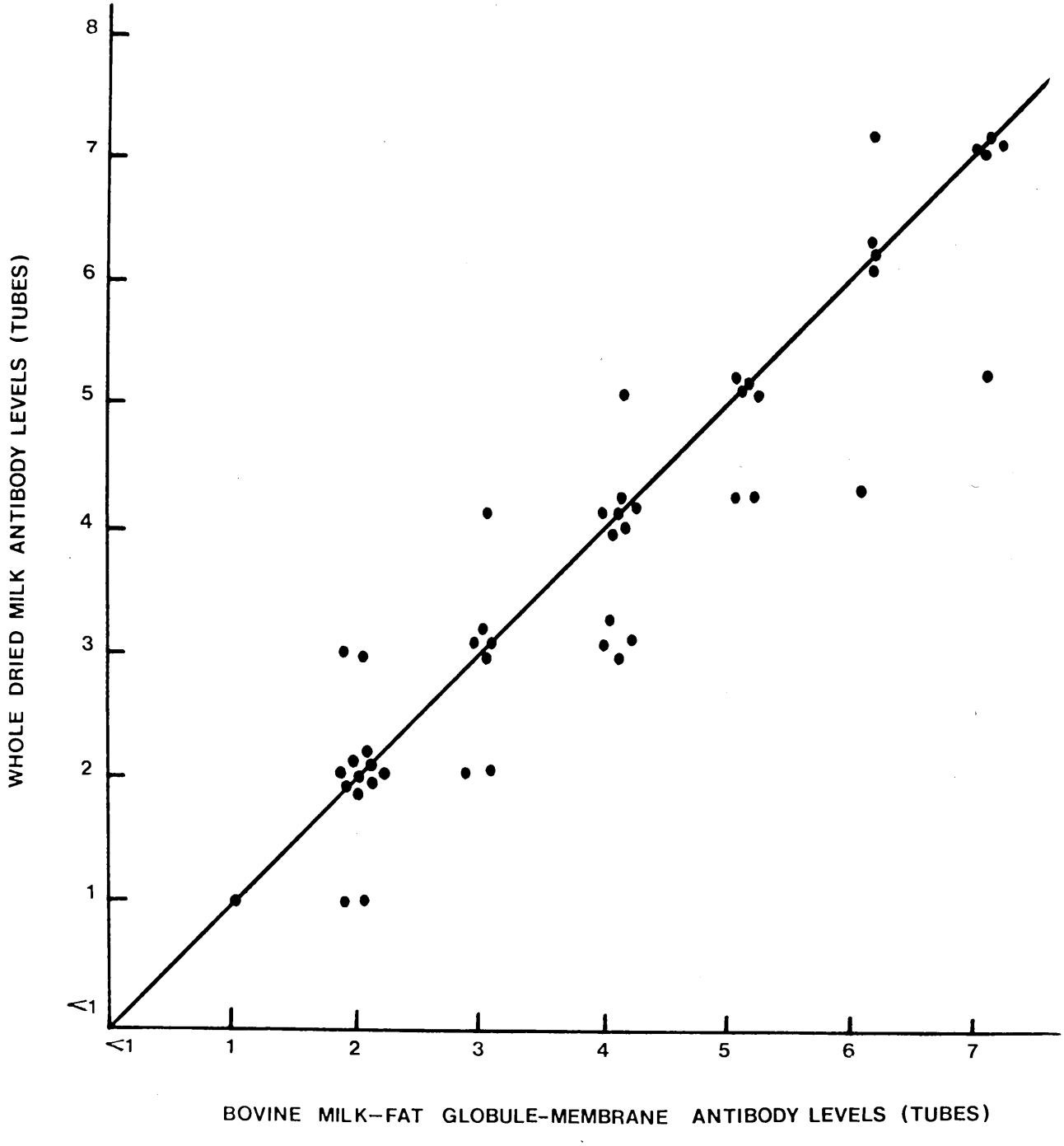

WHOLE DRIED MILK ANTIBODY LEVELS (TUBES)
8
7
6
5
4
3
2
1
<1
<1
1
2
3
4
5
6
7
BOVINE MILK–FAT GLOBULE-MEMBRANE ANTIBODY LEVELS (TUBES)

Fig. 51 demonstrating the correlation obtained when testing sera using the EIA method. The correlation 'r' obtained when comparing the antibody levels against whole dried milk and bovine-fat globule-membrane was 0.97 ($p < 0.001$).

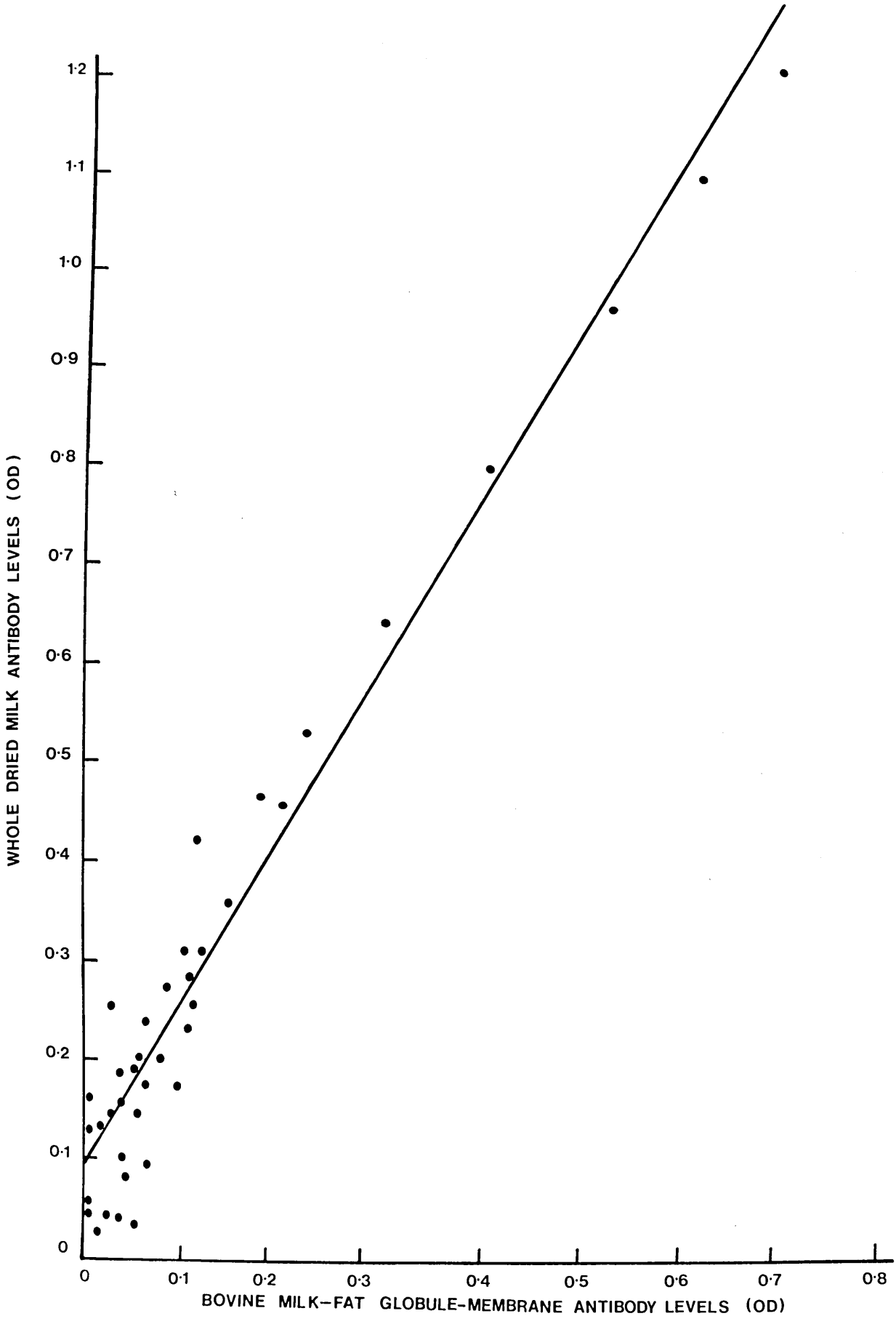

WHOLE DRIED MILK ANTIBODY LEVELS (OD)
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
BOVINE MILK–FAT GLOBULE-MEMBRANE ANTIBODY LEVELS (OD)

Table 19 exhibiting the results obtained by EIA and tanned red cell haemagglutination techniques. The antibody tested was milk-fat globule-membrane and the results are grouped in haemagglutination titres.

EIA results in OD	Haemagglutination results in titres	EIA results in OD	Haemagglutination results in titres
0.01	0	0.01	1/8
0.01	0	0.02	1/8
0.02	0	0.03	1/8
0.02	0	0.07	1/8
0.02	0	0.10	1/8
0.02	0	0.12	1/8
0.03	0	0.12	1/8
0.03	0	0.15	1/8
0.03	0	0.29	1/8
0.05	0	0.12	1/16
0.05	0	0.12	1/16
0.06	0	0.13	1/16
0.06	0	0.17	1/16
0.06	0	0.21	1/16
0.06	0	0.22	1/16
0.06	0	0.24	1/16
0.08	0	0.47	1/16
0.09	0	0.16	1/32
0.09	0	0.15	1/32
0.09	0	0.15	1/64
0.10	0	0.16	1/64
0.12	0	0.32	1/64
0.13	0	0.44	1/64
0.14	0		
0.14	0		
0.03	1/4		
0.04	1/4		
0.04	1/4		
0.05	1/4		
0.06	1/4		
0.06	1/4		
0.09	1/4		
0.10	1/4		
0.10	1/4		
0.10	1/4		
0.12	1/4		
0.22	1/4		

experiment was performed using a freshly prepared batch of milk-fat globule-membrane and the optimum dilutions of the various reagents were carefully monitored. The results returned from Flow laboratories and the comparison with the haemagglutination results are reported in Table 20. As can be seen an improved correlation was obtained, most of the haemagglutination titres of greater than $^1/_2$ exhibited a positive EIA titre.

8.4 Discussion

This chapter describes investigations attempting to identify and isolate the antibody previously referred to as "antibody to whole dried milk". It was found that casein, α-lactalbumin and β-lactoglobulin showed no correlation between antibody levels to these three main proteins of milk and that to whole dried milk. The absorption experiments confirmed that these three antigens were not related to or responsible for the "whole dried milk" antibody.

It was shown that a highly significant relationship existed between the levels of antibody to whole dried milk and to fresh whole milk. This excluded the possibility that the responsible antigen was related to denaturation of proteins during preparation of the dried milk or to any additives to the commercial product. The absorption experiments with cream, and the lack of correlation in results when fresh milk, with the cream removed, was tested, suggested that the antigen responsible resided in the cream portion of milk. This observation was confirmed when the results of sera tested with whole dried milk were compared with cells sensitised with washed cream. The correlation 'r' was 0.98 which was highly significant.

This knowledge that the responsible antigen was located in the cream portion of fresh whole milk formed the basis for further studies. Cream consists of fat globules. Borgmann and his co-workers (252), Harrison and Lunt (253) and Patton and

Table 20 demonstrating the comparison between the EIA and the haemagglutination method. The EIA results of < 0.100 were accepted as negative. When "absorption" experiments were performed on the positive sera, the levels were reduced to OD readings of < 0.100. This was considered to be equivalent to the "background" level. OD readings between 0.100-0.150 were reported as positive/negative. This level appeared to be an "intermediate range" where the haemagglutination results were negative or demonstrated a low titre. Most OD readings of > 0.150 correlated with a haemagglutination titre of $> {}^{1}/_{2}$.

In this table the negative results are reported as -, postive/negative as $\pm$, positive results as +, and "strong" positive results as ++.

The tanned red cell haemagglutination results are reported as titres.

OD	Titre	OD	Titre	OD	Titre	OD	Titre	OD	Titre	OD	Titre
±	0	-	1/2	-	1/4	-	1/8	+	1/16	++	1/32
-	0	-	1/2	+	1/4	+	1/8	+	1/16		
-	0	-	1/2	+	1/4	+	1/8	++	1/16		
-	0	±	1/2	+	1/4	+	1/8				
-	0			+	1/4	-	1/8				
-	0			-	1/4	+	1/8				
-	0			+	1/4	+	1/8				
-	0					+	1/8				
-	0										
+	0										
±	0										
-	0										
-	0										
-	0										
-	0										
-	0										
-	0										
±	0										
-	0										
+	0										
-	0										
-	0										
+	0										
-	0										
-	0										
±	0										
-	0										
-	0										
-	0										

Keenan (254), describe how the fat-globules are synthesised in the acinar cells of the mammary gland and, upon reaching a critical size, project into the duct pushing the acinar cell membrane around them. Eventually the cell membrane is separated and completely encases the milk-fat globule and is referred to as the "milk-fat globule-membrane". This is illustrated in Fig. 52. McKenzie (250) described this membrane found in cream as "highly antigenic and differs immunologically from all other recognisable milk proteins".

Sera tested against whole dried milk and also the bovine milk-fat globule-membrane showed a highly significant correlation in antibody levels ($r = 0.96$). Extensive cross-absorption studies confirmed that the antibody related to coronary heart disease, previously referred to as an antibody to "whole dried milk", is an antibody formed to an antigenic fraction of cream called "milk-fat globule-membrane". This finding made using the sensitised tanned red cell haemagglutination technique, was confirmed in collaboration with Flow Laboratories using the enzyme immuno-assay method. After further development work using this EIA technique, both Flow Laboratories and the author considered that an EIA method had been established with results comparable to those obtained by the tanned red cell haemagglutination technique which had provided the basic published data. In addition, this EIA method confirmed that the sera of some healthy adult males possessed an antibody to milk-fat globule-membrane. Flow Laboratories have now reported (255) that studies have shown that the structure of this antibody is consistent with the IgG class of immunoglobulins.

Patton and Keenan (254) describe the bovine milk-fat globule-membrane as being suitable for use as a "model system for the investigation of bio-membranes". Many similarities were observed by Dowben and his co-workers (256) between the behaviour of intact milk fat-globules and erythrocytes; one example is that they are both lysed by repeated freezing and thawing, sonication and by sapogenins. It is of interest that in 1674 van Leeuwenhoek (257) observed that butterfat in milk

Fig. 52 showing:-

(Top) The appearance of the principal microstructures involved in lactation. On the left is a single alveolus showing arrangement of lactating cells and (hollow) lumen area into which the milk is secreted. On the right is an enlarged version of a single lactating cell illustrating the mechanism of protein and fat secretion and showing the principal membrane system of the cell.

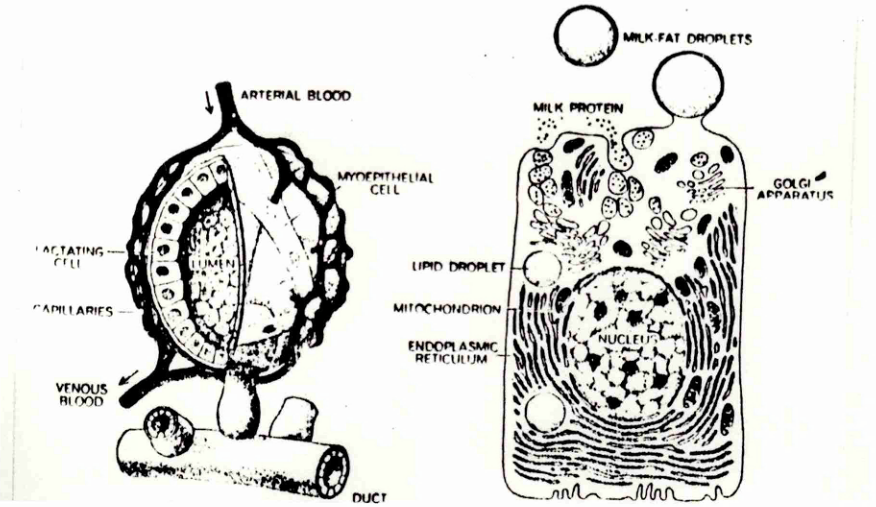
Reproduced by permission of the publishers. From Patton, S. (1969). Scientific American, 221, 58-68.

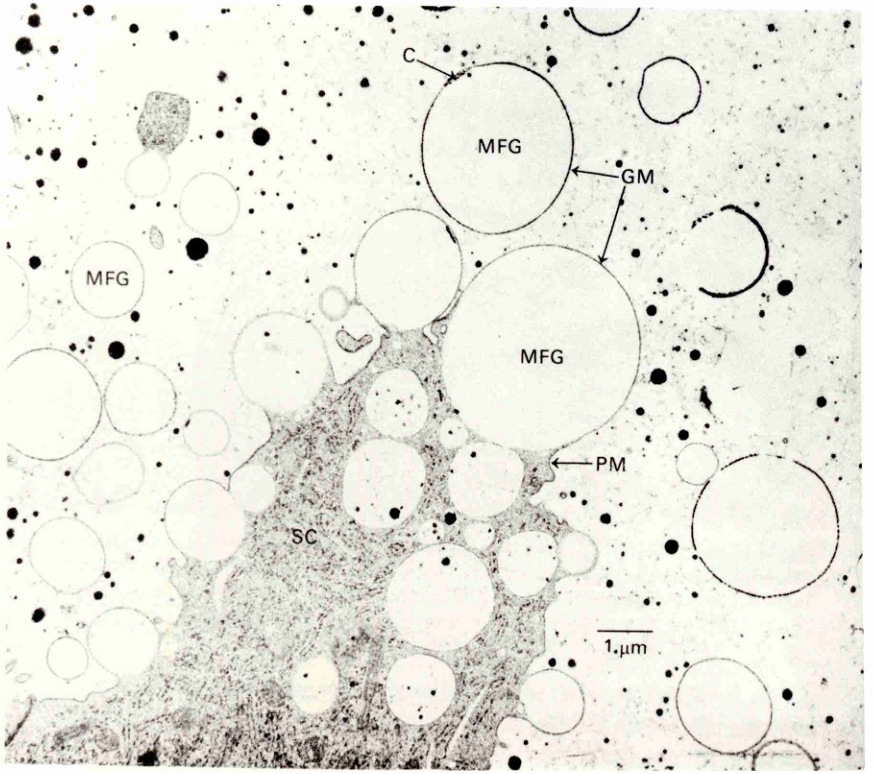
(Left) An electron micrograph of a section of goat mammary tissue showing milk fat globules (MFG). The globules approach the plasma membrane (PM) of the secretory cell (SC) and bulge through it. The globule forms a protuberance, which pinches off, forming a free milk-fat globule with a boundary membrane (GM). Small crescents of cytoplasm (C) are sometimes trapped in the globules.

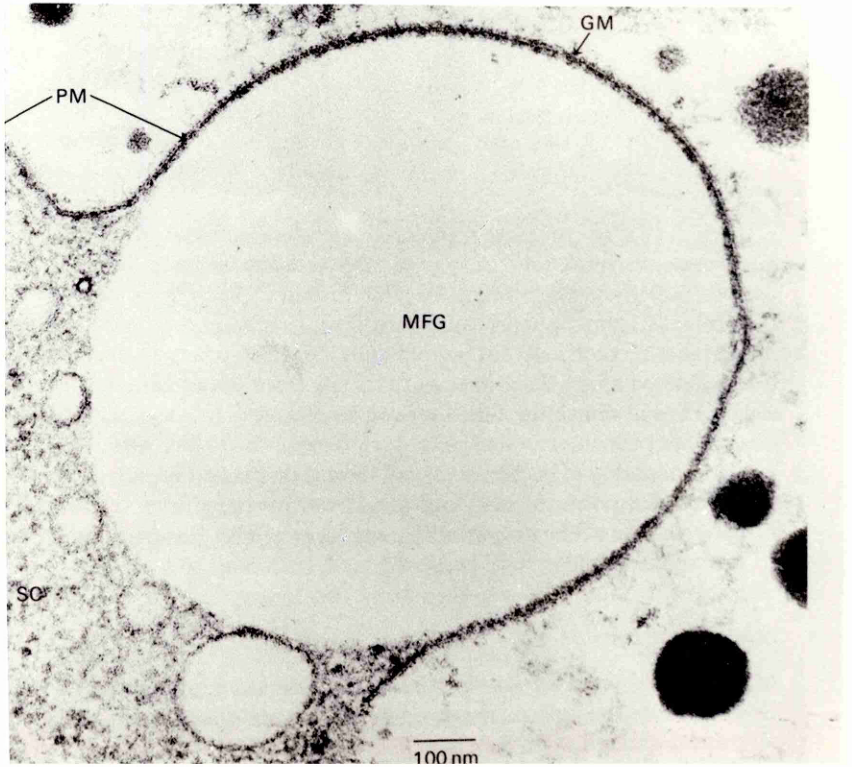
(Right) At a higher magnification, it can be seen how the plasma membrane (PM) of the secretory cell (SC) is continuous with the globule membrane (GM).

Reproduced by permission of the publishers. From Harrison and Lunt (1980), p.50 and 51 (253)

Also by permission of Dr. F. B. P. Wooding, Agriculture Research Council, Institute of Animal Physiology, Babraham who supplied the original micrographs.


ARTERIAL BLOOD
MYOEPITHELIAL CELL
LACTATING CELL
LUMEN
CAPILLARIES
VENOUS BLOOD
DUCT
MILK FAT DROPLETS
MILK PROTEIN
GOLGI APPARATUS
LIPID DROPLET
MITOCHONDRION
NUCLEUS
ENDOPLASMIC RETICULUM


C
MFG
GM
MFG
MFG
PM
SC
1 µm


GM
PM
MFG
SC
100 nm

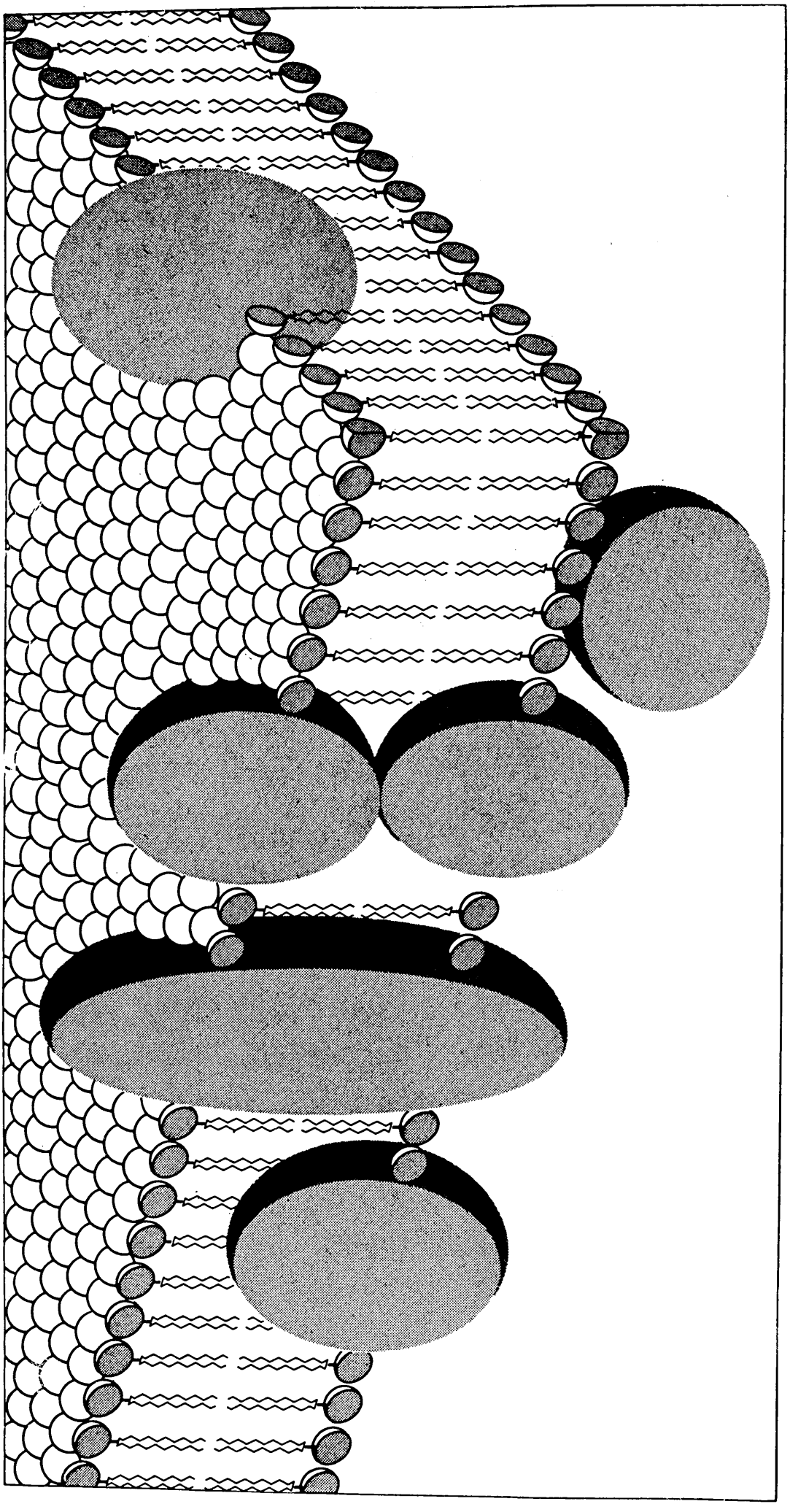
was dispersed as "granules" approximately 2μ in diameter and likened the microscopic appearance of a film of milk to that of blood. Electron microscopy studies (256) of milk-fat globule-membrane "ghosts" showed the structure of this membrane to be similar to other membranes and chemical analyses of the enzymes present showed similarities to those present in the erythrocyte membrane. The results of these and other tests, provided further data to confirm similarities in the properties and functions of the two membrane preparations. Dowben et. al. (256) after extensive investigations concluded that the bovine milk-fat globule-membrane was "a true biological membrane generically related to other membranes". The function and structure of the membrane has been described by Harrison and Lunt (253). These authors describe how the main function of the plasma membrane is to protect. It maintains a constant internal environment irrespective of changes that may occur outside. The plasma membrane is also involved in complex transfer systems where it controls the passage of nutrients into the cell and the removal of waste products from it. Plasma membranes have a basic structure of a double layer of lipid molecules with hydrophilic heads and hydrophobic tails. Also associated with the membrane are protein molecules which penetrate the membrane and others of which are embedded in one side or the other. This is illustrated in Fig. 53.

It is known that membranes throughout phylogeny are basically similar, Bjerrum (258) demonstrated cross-reactivity of antibodies to membranes against the same antigenic determinants in the membranes of other species. Comparable findings have been made by other workers (259-264). Bjerrum and Bøg-Hansen (265) commented that there was "a much closer immunological relationship between membrane proteins of different cell types than hitherto suggested".

Evidently the bovine milk-fat globule-membrane can be considered a biological membrane. This thesis, therefore, considers the possibility of cross-reaction between an antibody to this bovine membrane and antigenic determinants on

Fig. 53 showing the structure of the cell membrane. It is shown schematically according to current conceptions. The basic structure is a double layer of lipid (fat) molecules with their hydrophilic heads pointing outward and their hydrophobic tails pointing inward. Also associated with the membrane are protein molecules, seen here as large bodies some of which penetrate the membrane and others of which are embedded in one side or the other. Most of the movement of ions into and out of the membrane is by way of channels in the various proteins.

Reproduced with permission of the publishers. From Tosteson, D.C.(1981) in Scientific American, 244, p.132.



human membranes. Chapter 9 (page 93) describes investigation into the relationship between the antibody to bovine milk-fat globule-membrane and human platelets - both of which have been implicated in coronary heart disease.

CHAPTER 9

BOVINE MILK-FAT GLOBULE-MEMBRANE AND HUMAN PLATELET MEMBRANE

9.1 Introduction

In the previous chapter it has been discussed how antibodies developed against one membrane can cross-react against membranes of other species. This chapter describes experiments demonstrating that the antibody to bovine milk-fat globule-membrane reacts with human platelets.

9.2 Materials and Methods

9.2.1 Production of antibody to bovine milk-fat globule-membrane. The bovine milk-fat globule-membrane was prepared, using sterile precautions, as described on page 85. Antibody to this preparation was raised in laboratory rabbits. Pre- and post-injection rabbit sera were tested for the presence of antibody to bovine milk-fat globule-membrane using haemagglutination and EIA methods.

9.2.2 Preparation of platelets. Platelets were obtained from ten normal healthy males and washed in 0.06M phosphate buffer saline (pH 7.2). After washing, the individual preparations of washed platelets were pooled. For testing, the final count was adjusted to 250 x 10^9/l

9.2.3 Reactivity between antibody to bovine milk-fat globule-membrane and platelet membrane. This was demonstrated using (1) the EEL aggregation system, (2) a fluorescence method.

1. The procedure followed was similar to that described for ADP aggregation on page 28 . One ml aliquots of platelet

suspension were placed in tubes supplied with the EEL aggregation meter. After one minute, 0.1ml samples of the rabbit sera were added and the aggregation patterns recorded.

2. The fluorescence technique used was based on the method described by Von Dem Borne et. al. (266). To 0.1ml aliquots of pooled platelets, 0.1ml samples of rabbit sera were added. These were incubated at 37°C for 30 mins. After washing, they were treated with anti rabbit immunoglobulin FITC. After further incubation and washing, examination for fluorescence was made using a Leitz Dialux 20 Microscope (Leitz Instruments Ltd., 48, Park Street, Luton).

9.3 Results

Both the pre- and post-injection sera were tested in parallel on the aggregation meter. It was found that volumes down to 0.1ml of the post-injection specimens induced strong platelet agglutination. The same volume of the pre-injection sera exhibited no agglutination. This is illustrated in Fig. 54. The post-injection sera showed marked membrane-bound fluorescence and also caused clumping. The pre-injection sera showed slight background fluorescence only and the platelets remained discrete.

The tanned red cell haemagglutination technique showed that the pre-injection sera possessed antibodies to a titre of $^1/_8$ and the post injection specimens a titre of approximately $^1/_{4000}$. The EIA results showed optical density readings equivalent to the haemagglutination titres. Extensive cross-absorption studies showed that an antigenic solution of whole dried milk reduced the titre of the antibody to bovine milk-fat globule-membrane to zero and vice-versa.

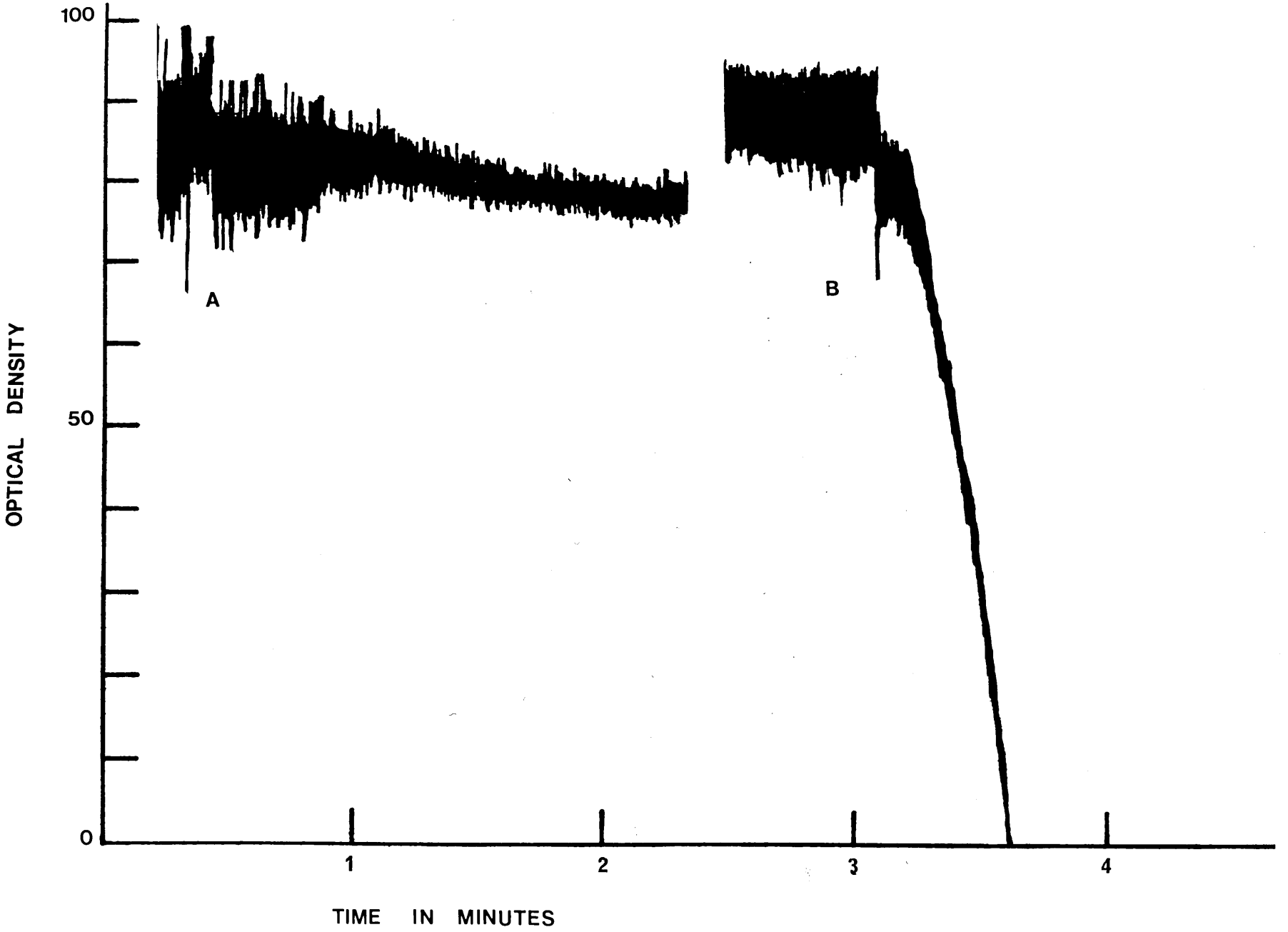
9.4 Discussion

The investigations in this chapter describe work performed

Fig. 54 demonstrating platelet response after the addition of rabbit sera.

Tracing 'A' shows a lack of response to the pre-injection sample of rabbit serum.

Tracing 'B' reflects the agglutination observed after the addition of the post-injection specimen.

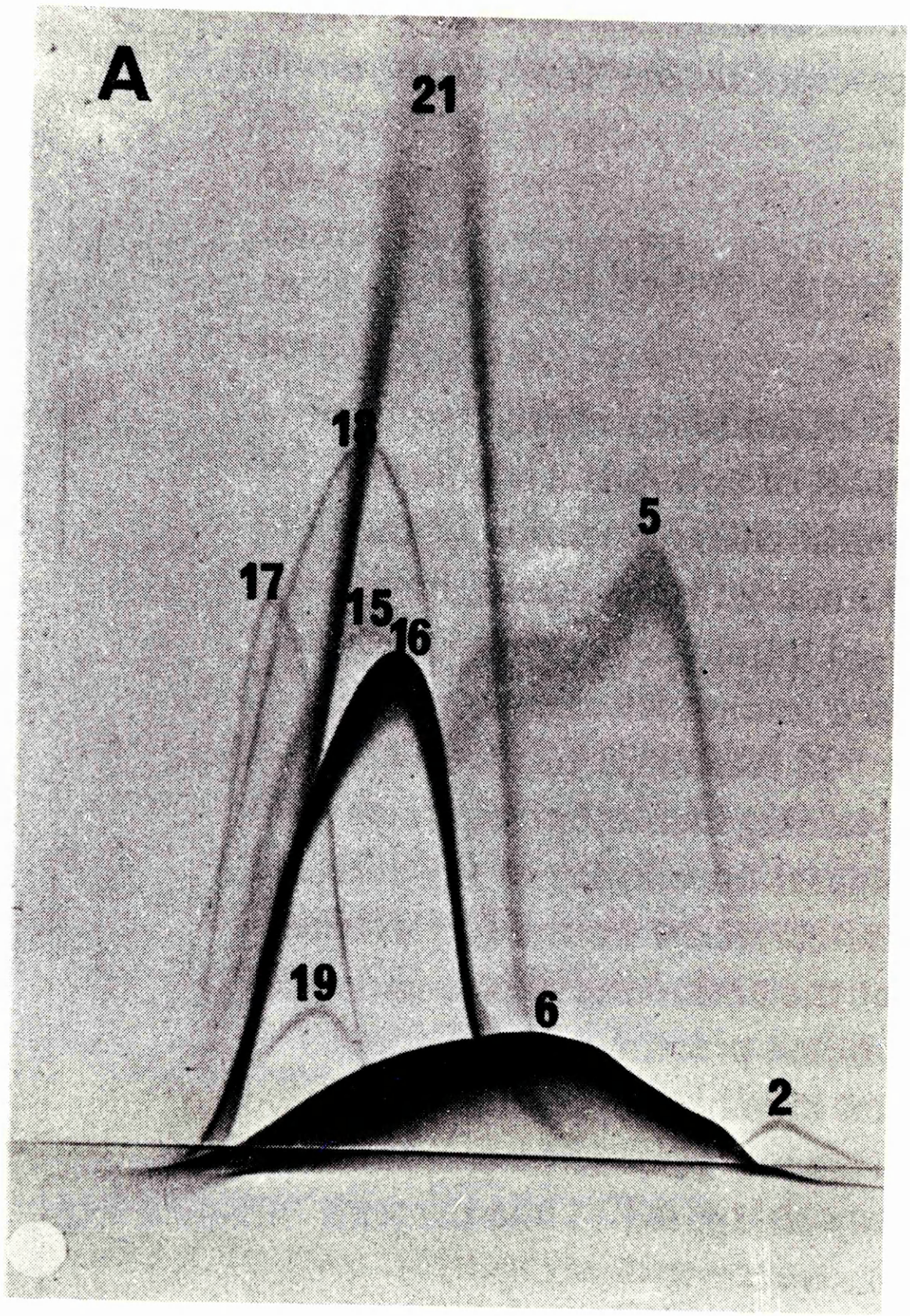

100
50
0
OPTICAL DENSITY
A
B
1
2
3
4
TIME IN MINUTES

with antisera, raised in rabbits, and establish that antibody to bovine milk-fat globule-membrane reacts with human platelet membrane. The theory of cross reactivity between membranes of different species has been described in Chapter 8, page 92. There is published evidence that antigenic determinants of human platelets membrane may bind with antibodies formed against bovine milk-fat globule-membrane. Thus, Bjerrum and Bøg-Hansen (265) describe how antibodies can be used for verifying that the same molecular structures (antigenic determinants) exist on the membranes of different cell types. When using cross-immunoelectrophoresis of berol-solubilised membranes and appropriate antisera, precipitation lines are obtained. These can be standardised and identified using a reference method. Fig. 55 demonstrates the precipitation lines obtained when testing solubilised human erythrocyte membrane against antibodies to this membrane. The numbers indicate and are used to identify the proteins involved. Although these numbers are allocated arbitrarily and the identity of the proteins are unknown, when the same method of identification is employed, and experimental conditions are identical, they should represent the same antigenic determinants, irrespective of the membrane system or species involved.

Bjerrum and Bøg-Hansen (265) demonstrated that antibodies raised in rabbits against bovine milk-fat globule-membrane cross-reacted with some of the major proteins of the bovine erythrocyte membrane. These were identified as numbers 5, 6 and 16. Immunoelectrophoresis studies of solubilised human erythrocyte membrane and antibodies to bovine erythrocyte membrane demonstrated that these three determinants were also common to these two membranes (258, 265). This suggests bovine milk-fat globule-membrane and human erythrocyte membrane share three common antigenic determinants; 5, 6 and 16. Hagen and his co-workers (267) extensively examined human platelet membrane using the same method. They found that these three antigenic determinants were also present on the platelet membrane. It therefore follows that antibody to bovine milk-fat globule-membrane should also react specifically with the same determinants shown to be present on the human platelet membrane.

Fig. 55 showing crossed immunoelectrophoresis of solubilised human erythrocyte membrane proteins and an antibody to this antigenic preparation. The numbers on the precipitation lines denote particular proteins.

Reproduced with permission of the publishers. From Bjerrum, O. J and Bøg-Hansen, T. C. (1975), p.388 (265).


A
21
18
5
17
15
16
19
6
2

Another possible means of activity is now considered. It has been demonstrated that antibody to bovine milk-fat globule-membrane is a lgG class immunoglobulin (255). When an immunoglobulin is treated with papain, the molecule is split into three parts (268). Two of these are identical and capable of combining specifically with the related antigen (269). These fragments are termed Fab (Fragment antigen binding). The third fragment crystallises after purification and so is referred to as Fc fragment (Fragment crystallizable). This fragment has not the ability to combine with the antigen. However, the Fc fragment can react with "Fc-receptors" present on certain cell membranes. This phenomenon could also explain the reaction between antibody to bovine milk-fat globule-membrane and platelet membrane. But such an alternative explanation for the phenomenon demonstrated would not detract from its significance in the reaction between these two membranes.

In summary, work described in this chapter has demonstrated that antibody to bovine milk-fat globule-membrane reacts with human platelet membrane. This forms the basis for further discussion which will be included in the "General Discussion" section of this thesis.

CHAPTER 10

GENERAL DISCUSSION

The findings presented in this thesis confirmed the involvement of platelets in the development of atherosclerosis and coronary heart disease (CHD). Emphasis was placed on the role of "active" platelets in these conditions. It was explained how individuals with a higher percentage than the normal 20-25 per cent "active" platelets could be susceptible to atherosclerosis. A test was developed, based on the "disaggregation ratio" after the addition of a "critical dose" of ADP, and was used in a "blind" survey performed in conjunction with the Medical Research Council Epidemiology Unit at Cardiff. Analyses of the results showed that abnormal results were demonstrated in approximately 90% of MI patients who had suffered the infarction at least twelve months previous to the test. The normal population exhibited approximately 19% abnormal results and the hospital based controls 13%. This test therefore offers a means of identifying individuals who have a predisposition to coronary heart disease.

The findings of these investigations need to be considered in relation to the two theories which are current in this particular subject. The first being the increased platelet activity is an effect of atherosclerosis and the second is that it plays a role in the initiation of atherosclerosis and coronary heart disease.

Examination of the data highlights one of the problems of investigating this disease. The presence of abnormal results in approximately 19% of the normal population demonstrates that the abnormality in the confirmed MI patients is not likely to be secondary to the infarct. Atherosclerosis is one

condition where the patients show no clinical evidence whatsoever of its presence (142). Under these circumstances it is difficult to define "normal" controls because "healthy" males may have atherosclerotic arteries. Myocardial infarction is only a clinical manifestation of coronary heart disease (90). This thesis therefore examines the data presented for evidence of the "active" platelets being the result of coronary heart disease against them being the cause.

The abnormal results in 90% of the confirmed MI cases demonstrate that most confirmed patients have a higher than normal number of "active" platelets present in their circulation. Although this is highly significant, it does not aid in elucidating the question of their presence being primary or secondary. It is therefore important to examine the abnormal results in the approximately 19% of the "normal" population. These results may have an influence on the important question of cause and effect.

Those investigated were all healthy adult males as determined by the Medical Research Council staff. It is noted that the percentage of abnormal results found in this normal population approximates that of men reported to be affected by clinical or sub-clinical coronary heart disease (205).

This data may well suggest a predisposition to CHD in some apparently normal healthy subjects. This could be due to premature build-up of atheromatous lesions caused by factors such as life style, heredity, possibly premature ageing or to others of unknown aetiology. Similarly, it could be argued that the presence of these platelets could be primary and are actively involved in initiating the process which terminates in clinical coronary heart disease. Their presence could reflect the ability of some individuals to produce antibodies against certain immunological factors

that influence platelet turnover (see page 20). Genetical factors could conceivably influence and result in an increased production of thrombopoietin and subsequently in an increased turnover of platelets.

The data presented in the platelet section offers no conclusive evidence to support the two theories discussed. Therefore, the more controversial theory that the "active" platelets play a role in the development of atherosclerosis cannot be excluded at this stage. This should not detract from the fact that the test developed was capable of detecting the majority of confirmed MI subjects and could play an important role in detecting individuals predisposed to coronary heart disease.

The evidence presented to elucidate the exact role of "active" platelets appears to attribute equal importance to both the cause and effect theories. To develop the theme of crucial importance to our understanding of the primary or secondary nature of these platelets, investigations were performed on the immunological aspect of coronary heart disease.

This section described investigations performed to elucidate the role of antibodies to whole dried milk in CHD and the distribution of other food antibodies in the population. It was found that the antibody which was originally related to coronary heart disease was produced in response to an antigen present in the cream portion of milk. It was subsequently demonstrated that this antigen resided in the bovine milk-fat globule-membrane, of bovine cream.

Using the EIA method (page 85), it was confirmed that a relationship existed between the antibody to whole dried milk and bovine milk-fat globule-membrane. The correlation between the haemagglutination and the EIA methods was satisfactory, considering that the methods utilised completely different systems of antibody detection. A similar method, but using isotope (^{125}I) to determine antibody level has

subsequently been developed in the Biochemistry Department of the West Wales General Hospital. This test was introduced by Mr. N. M. Deadman and Mr. J. D. Evans of the Isotope Unit. The results obtained, using this isotope method, confirmed all the observations made by Flow Laboratories; the relationship between the antibody to whole dried milk and the bovine milk-fat globule-membrane was again confirmed.

The development of an automated system (see page 63) enabled the investigation to demonstrate that normal males and females produce antibodies to milk, egg and gluten. No response was shown, using this method, to meat either cooked or raw form. It was discovered that the antibody levels to egg and gluten demonstrated a similar distribution in the male and female populations. But antibodies to milk did not, for more females possessed antibodies to whole dried milk and the levels were also higher. Although the milk antibody level is higher, the incidence of coronary heart disease is lower in the younger female than in males of the same age group. This aspect has been considered in the text (page 78) and evidence exists which suggests that this apparent protection can be attributed to oestrogen (245).

The discovery that the antibody implicated in atherosclerotic coronary heart disease is directed towards a fraction of cream called bovine milk-fat globule-membrane offers an explanation of why this work on milk antibody and coronary heart disease was refuted by certain workers (270, 271). One (270) used "fat-free" milk and the other (271) "National Dried Milk", a preparation which was low in its contents of cream. Oster (272), using an antigenic preparation consisting of whole dried milk, independently confirmed the original work of Davies et. al. (178).

One observation was made which is important in relation to the use of the tanned red cells haemagglutination technique for estimating membrane antibodies. From January to September, 1982, the isotope method has given consistent

results. The haemagglutination technique however, demonstrated unsatisfactory results with the standard sera during the winter months, returning to the expected values during the spring. The volume and the consistency of the membrane preparations were strikingly different during these two seasons. The unsatisfactory variability in the results with the tanned red cell haemagglutination technique was probably due to the physical state of the membrane and its capability of being absorbed on the surface of the tanned red cells. This observation may also be of dietary significance. It was fortuitous that the original observation, describing the relationship between the antibodies to whole dried milk and bovine milk-fat globule-membrane, was made during the summer months. In the author's laboratory future work on membranes will be performed using the enzyme (or isotope) immuno assay method.

Published evidence describes the bovine milk-fat globule-membrane as a "true biological membrane" (256). It is also known that cross-reactivity exists between the membranes of different species (258-264). An experiment was designed to investigate whether any reactivity occurred between an antibody to bovine milk-fat globule-membrane and the platelet membrane. It was discovered that reactivity did occur. It is important to consider the significance of such a reaction and the possible mechanism by which such an antibody could attach itself to the platelet membrane. It has been described in the text how an antibody produced to bovine milk-fat globule-membrane contains antibodies to antigenic determinants 5, 6 and 16. These determinants were shown by Hagen and his co-workers (267) to be present on the human platelet membrane. Relevant to this, Hagen et. al. (267) showed that the human platelet membrane contains twenty antigenic determinants. Six of these were shown to be glycoproteins, the main one present, which was "probably exposed at the outer platelet surface" was demonstrated to be number 16. Therefore, if this is the case, antigenic determinant is easily accessible

to the effect of circulating antibodies to bovine milk-fat globule-membrane. The cross-reactivity between these two membrane preparations could be demonstrated using the cross immuno electrophoretic method, which would involve testing the antibody to bovine milk-fat globule-membrane against solubilised platelet membrane.

The possibility of this antibody reacting via the Fc receptor (page 96) should also be considered. This could be performed by digesting the antibody with pepsin (273). As a result the Fc fragment is destroyed, but the Fab fragments remain capable of reacting specifically with the antigen involved. The influence in the test of a non-specific "anti-human" antibody can be disregarded as the test was controlled with pre-injection samples from the rabbits. However, the action on the platelets by milk-fat globule-membrane antigen/antibody complexes warrants consideration but the presence of these can be demonstrated by many techniques routinely used for the detection of complexes.

It is now evident that a percentage of the normal male population, and a greater percentage of confirmed myocardial infarction patients, have an excess of "active" platelets. There is also a correlation between confirmed MI patients and the presence of antibody to bovine milk-fat globule-membrane. It has been demonstrated that this antibody will aggregate platelets. One explanation for the presence of the increased percentage of "active" platelets can therefore be the reaction between the antibody to bovine milk-fat globule-membrane and the human platelet membrane.

Such a reaction will inevitably be followed by their removal from the circulation (137, 143-153) resulting in increased platelet turnover and explain the excess "active" form present in the blood. It therefore follows that the presence of an increased number of "active" platelets in the circulation of the normal controls, points to the possibility that they are actively involved in the process terminating

in clinical coronary heart disease.

Relevant to the investigations described in this thesis, is the work of Butler and Osking (274). These workers have shown reactivity between an antibody to bovine associated mucoprotein and various human tissues. This mucoprotein is a constituent of the milk-fat globule-membrane. This work (274) involved cancer, IgA deficiency and auto-immunity. Platelets were not tested in this experiment, but the results showed reactivity between an antibody to a portion of the bovine-milk fat-globule membrane and human epithelial membrane antigen and other human cell antigens. Antibodies to human epithelial membrane has also been shown to react with human milk-fat globule-membrane-derived glycoproteins (275).

This thesis has demonstrated that interaction between an antibody to bovine membrane and human platelets may play a role in the development of atherosclerosis. The phenomenon by which the body is stimulated to produce antibody against "self", resulting in a clinical condition, should be considered. Group A streptococcal antigen has been shown to cross-react with the myocardium (276, 277). Some workers believe that group A beta-haemolytic streptococci of types 12, 4 or 49 could have a similar action on the kidney (276). Welsh and his co-workers (278) found cross-reactivity between ankylosing spondylitis, HLA-B27 and Klebsiella. There are 13 antigenic determinants (258) on the bovine milk-fat globule-membrane. It will be highly significant to discover whether the body produces antibodies to all these and the cross-reactivity discussed is caused by a small number of antigens which happen to be common to the bovine milk-fat globule-membrane and the platelet membrane.

Certain conclusions have been drawn from the work described in this thesis. It is important to consider possible new lines of investigations. These are outlined below:-

1. Comparison between the ADP disaggregation patterns and

antibody levels to bovine milk-fat globule-membrane using the isotope immuno-assay method.

2. Electron microscopy studies (279) to ascertain whether the platelets circulate in the "active" form or whether this activity is only important after they have been stimulated to undergo their "release reaction".

3. Absorption of the bovine milk-fat globule-membrane antigen into the blood stream utilising the inhibition method and using the isotope immuno-assay technique.

4. Isolation of the fraction of this antigen in relation to coronary heart disease patients.

5. The immunological theory could be pursued by measuring the various complement levels and also circulating antigen/antibody complexes.

6. Platelet material can be added to trays (280) and the isotope immuno-assay method performed to detect antibody in sera directed against platelets. This sensitive method could demonstrate a cross reactivity in some normal subjects.

In conclusion, this thesis describes the significance of detecting the increased percentage of "active" platelets in coronary heart disease. Evidence is given to demonstrate how antibodies to bovine milk-fat globule-membrane can result in the appearance of such platelets. It is therefore proposed that interaction between membranes should be considered as one important contributory factor in the development of atherosclerosis and coronary heart disease.

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The Central Office of Information recently sent a team to the laboratory to make a film of the research work into coronary heart disease.

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